"*Brachyspira hampsonii*" associated diarrhea in pigs: virulence assessment and host-pathogen interactions

Submitted to the College of Graduate Studies and Research of the University of Saskatchewan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Veterinary Microbiology at the University of Saskatchewan.

By

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ABSTRACT

This thesis aimed to verify the causal association between *"B. hampsonii"* and the re-emergence of mucohaemorrhagic diarrhea in North American swine farms, to investigate the role of the intestinal microbiome as a predisposing factor for infection, to develop a porcine colon *in vitro* culture model and to apply this model in investigating early host-pathogen interactions.

Two infection trials were conducted to determine the pathogenicity of *"B. hampsonii"* clade II and clade I. Weanling pigs were divided into control (n=6) and inoculated (n=12) groups. In each trial, pigs were inoculated with *"B. hampsonii"* clade II (tissue homogenate or pure culture) or clade I (pure culture) or sterile culture media. Animals were monitored for clinical signs of diarrhea and upon observation of bloody diarrhea they were necropsied for characterization of lesions. Fecal shedding of *"B. hampsonii*" was monitored throughout the trials using culture and quantitative real-time PCR. Pre and post-diarrhea fecal samples from the clade II infection trial were used to study the microbiome response to *"B. hampsonii"* infection and to determine if pre-inoculation microbiome composition differed between pigs that did or did not develop clinical disease. For *in vitro* model development, numerous factors associated with explant survivability in culture were investigated to develop a protocol for culture of porcine colon explants. The optimized model was used to study the first 12 hours of *"B. hampsonii"* clade II interaction with the host using a combination of histopathology and gene expression analysis.

Pigs inoculated with *"B. hampsonii"* clade I (9/11) and clade II (9/12 and 8/12 in the tissue homogenate and pure culture experiments, respectively) developed mucohaemorrhagic diarrhea and colitis within 14 days of inoculation. In all trials, mucohaemorrhagic diarrhea was significantly more common in inoculated pigs than controls. No significant differences in

richness, diversity or taxonomic composition distinguished the pre-inoculation microbiomes of affected or unaffected clade II inoculated pigs. After the development of diarrhea, the fecal microbiome of diarrheic pigs was more dense and had a had a lower Bacteroidetes:Firmicutes ratio when compared to inoculated but unaffected or control pigs. Cultured porcine colon explants displayed differentiated epithelium and crypts after 5 days in culture, while expressing GAPDH at a constant rate. For explants to thrive *in vitro* our results suggested the use of distal spiral colon, processed immediately after euthanasia, and cultured in an oxygen-rich gas mix with air-liquid culture interface in media containing antibiotics and antifungals. Explants exposed to *"B. hampsonii"* for 12 hours had a greater number of necrotic cells and thicker catarrhal exudate than control explants. Interaction of spirochaetes with the epithelium, necrotic cells and crypts was visible under optical microscopy, and a trend of increased expression of IFN-γ and e-cadherin in inoculated explants relative to control explants was observed.

Taken together, results of this thesis demonstrate that *"B. hampsonii"* causes mucohaemorrhagic diarrhea in pigs and modulates their intestinal microbiome. The development of an *in vitro* infection model that replicates *in vivo* features facilitated the observation of the initial events in *"B. hampsonii"* interaction with the colon. When explants were exposed to *"B. hampsonii"* similar histological lesions to *in vivo* were observed. This system provides a powerful model for future studies of the pathogenesis of *"B. hampsonii*" and other enteric pathogens of pigs.

ACKNOWLEDGEMENTS

"When eating bamboo sprouts, remember the men who planted them."
Chinese Proverb

Through this journey I have come to the realisation that I am made from small parts of the ones I shared my life with, I am collection of all of you. Thank you.

For her patience, her will to share her knowledge, her passion for science, her ability to teach and to teach how to teach, her patience (yes, again), her office door being always open, her ability to do things in record time (specially over the weekends!), her *guidance*! I am certain that keeping me in the path was not an easy task. Thank you, Janet. Thank you for not being my supervisor, but my model and inspiration.

For his skepticism, his incredible ability to make me question myself when I was 100% sure of my answer, his knowledge that was shared, his advices and directions, his willingness to read my work within short notice. Thank you John for helping me navigate the unforgiving seas of graduate school. Thank you for not being my supervisor, but my model and inspiration.

To my parents and brothers, you are my foundation. I'm sorry I was away and missed many important moments. Thank you for allowing me to do this and supporting me.

Friends, until now you may not know that I would not have lasted this long without you. Countless advices, many adventures. Keeping me entertained and motivated during these years made it way easier than I ever expected it to be. To my Canadian family, Silvia, Marcia, Thiago, Fernanda, Roberto et al, Klebér et al. (this was not organized in any special way), foi com vocês que eu recarreguei as baterias (por exemplo, nos inúmeros churrascos e jantares). Muito obrigado por me aturarem, me acolherem e compartilharem de suas famílias comigo. Meu mais que muito obrigado, meu *PQP* MUITO OBRIGADO MESMO!

To the Hill Lab & aggregates: Atul, Bonnie, Champika, Isha, Teenus, Matt, Yanyun, Chrissie, Joe, Lisa, Aline, Jason, Mo, Niradha, Noreen, Arinjay, Vikram, Michelle, Linda and Lana: thank you for your help, chats, frustrations (listening to mine and sharing yours) and achievements! It was a true learning experience!

To the Loewen lab, Dr. Wolfgang Koester, Dr. Michael Dame, ACU, GMP and caretakers – thank you for your assistance. Also, thank you for the experience exchange, culture advice, animal handling and helping with last minute media preparation or room cleaning! Not a single experiment would have been done without you.

There are for sure many others who should be included here and I apologize for not citing all of you, but be sure I'm thankful for the piece of you I now carry.

For my partner-in-crime, my gratitude in words is not enough. I hope to give it back to you through actions during our future together.

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1 INTRODUCTION AND LITERATURE REVIEW

1.1 Emergence of a swine dysentery-like syndrome

Brachyspira associated diarrhea cases became rare in Canadian herds during the 1990s and early 2000s (Harding, J.C.S., 2011, personal communication). During this period, effective biosecurity procedures were enforced in commercial swine production systems. Measures to control fomite trafficking including geographical isolation, entrance showers, farm dedicated vestments, equipment and vehicles, may have contributed to decreasing the number of cases and control of the disease. Additional procedures such as early weaning of piglets, "all-in/all-out" facilities and three-site systems helped to limit contact with infection sources. In addition, diet composition changes may have played a role in limiting spirochaetal growth. However, during the first decade of the current century Canadian and US producers have faced the re-emergence of a SD-like syndrome.

The first report described clinical cases from commercial farms in 2009 that resembled SD, but diagnostics failed to detect *B. hyodysenteriae* in samples (Harding et al., 2010a). Other authors from the USA also reported such findings, and further investigation of isolates obtained from clinical cases followed. *Nox* gene sequences from such atypical strains provided the first evidence for a novel agent, followed by phenotypic description of the bacteria. Finally, pure culture inoculations of mice and pigs and molecular characterization of isolates led to the proposal of an emergent new pathogenic species, provisionally called *"Brachyspira hampsonii"*,

divided into clades I and II (Burrough et al., 2012a; Burrough et al., 2012b; Chander et al., 2012). The possible association of "*B. hampsonii"* with a syndrome undistinguishable from SD brings a range of challenges for producers and veterinarians involved in pork production. As for any other emerging pathogen, understanding the disease process and host-pathogen interactions is essential for development of effective diagnostics, prevention and control methods. Infection models, both *in vivo* and *in vitro*, set the foundation to achieve these objectives as they mimic clinical outbreaks under a controlled environment.

1.2 The swine large intestine

1.2.1 Anatomy

Pigs (*Sus scrofa domestica*) are a domesticated species of mammalian hindgut fermenters commercially raised for meat and fat production. Their large intestine, comprising the caecum and colon, is responsible for production of short-chain fatty acids that contribute up to 25% of the animal's energy requirements (Rerat et al., 1987). Classical anatomical description of the large intestine includes the cecum, ascending, transverse, and descending colon, rectum and anus, with all, except for the anus, being found in the abdominal cavity (Dyce et al., 2009; Sisson et al., 1975). The cecum is an intestinal modification of herbivores and omnivores. It consists of a blind sac laying on the dorsocranial aspect of the left flank, extending ventrally, caudally and medially to the spiral colon in a way that the blind end of the sac meets the abdomen near the *linea alba*. Its surface has three distinctive smooth muscle bands longitudinally oriented, the

taeniea ceci, and three saculations (*haustrae)*. The colon has initially the same diameter as the cecum and is formed by several saculations. It is connected to the dorsal abdominal wall by the mesentery, which attaches between the kidneys. Anatomically, the colon is divided into ascending, transverse and descending sections (Sisson et al., 1975). The ascending colon is unique in swine due to the helix shape it assumes. It is commonly referred to as the spiral colon, given its greater than 360° rotation developed early in embryogenesis (Dyce et al., 2009). The colon also displays *taeniea*, but only two bands of smooth muscle are observed. The transverse colon crosses from the end of the spiral loop to the left side of the abdomen. It continues caudally into the pelvic cavity as descending colon, which opens into the rectum, rectal ampulla and finally the anus, the exterior intestinal opening.

1.2.2 Vascularization

The large intestine is well vascularized through a web of vessels within the mesentery. More precisely, branches of the superior mesenteric artery and inferior mesenteric artery, which are branches of the internal iliac arteries, are responsible for supplying the caecum, colon and rectum with blood. Distal segments of the rectum are also supplied by the middle and inferior rectal arteries. The portal system is the main path for venous blood out of the large intestine, whereas a minor portion of blood is drained by systemic veins *via* the hemorrhoidal plexus. Lymphatic channels running along the blood vessels drain the lymph towards the intestinal trunk and finally to the chyle cistern (Sisson et al., 1975).

1.2.3 Innervation

The colon is innervated by two different sources of nerves: the extrinsic and intrinsic nerves. Extrinsic innervation arises from the autonomic nervous system branches of sympathetic and parasympathetic nerves. Intrinsic nerves originate from the enteric nervous system.

Parasympathetic nerves are associated with excitatory effects on colonic motility. The principal neurotransmitters present in these fibers are acetylcholine and tachynins such as substance P. Branches of the posterior division of the vagus, the tenth cranial nerve, are responsible for innervating the proximal colon and are found along branches of the superior mesenteric artery (Sisson et al., 1975). The distal colon is innervated by branches of the second, third and fourth sacral nerves. These travel into the peritoneum to join the inferior hypogastric plexus where they branch into the colon submucosa to ultimately synapse at the plexi of Auerbach and Meissner (Dyce et al., 2009).

The sympathetic system acts in opposition to the parasympathetic system by decreasing colonic perilstasis, secretion and splanchnic blood flow. However, it is excitatory to sphincter muscles such as the ileocecal sphincter and the internal anal sphincter. Innervation is supplied by branches of T1-T12 and L1-3, and the main neurotransmitter in these fibres is norepinephrine (Feldman et al., 2015).

The enteric nervous system is a separate entity from the central nervous system and the spinal cord. This system is to some extent self-regulated, being largely responsible for colonic motility.

However, the sympathetic and parasympathetic nerves that infiltrate the large intestine may influence it. Cells within neuronal plexi are located between muscle layers (plexus of Auerbach) or within the submucosa (Meissner's plexus). Control of colonic motor function is still poorly understood, but it seems to coordinate with an arch reflex elicited by mechanical or chemical agents that activate primary afferent neurons in the mucosa. The stimulus travels to an enteric motor neuron *via* enteric interneurons, producing either an excitatory or inhibitory effect depending on the luminal situation (Christl and Scheppach, 1997; Sisson et al., 1975).

1.2.4 Histology

1.2.4.1 Mucosa

The large intestine is composed of a specific set of tissues that together give the organ its characteristic functional features. Beginning with the luminal aspect, it may be divided into mucosa, submucosa, radial and longitudinal muscularis and serosa. The mucosa is formed by epithelial cells in direct contact with luminal contents. The superficial epithelial layer (*lamina epithelialis mucosae*) is an heterogeneous population of mature epithelial cells that have finite lifetimes and exfoliate into the lumen, requiring constant renewal (Saunders et al., 1975). The epithelium is a polarized, single-cell columnar epithelium, which also includes goblet, enteroendocrine, Paneth and M cells. Lymphocytes and occasional eosinophils may be found between the surface epithelial cells, while neutrophils are not normally present at this site (Levine and Haggitt, 1989). Columnar epithelial cells (colonocytes) account for up to 95% of the cell population of the *lamina epithelialis mucosae*. The epithelium presents with microvilli and a

glycocalyx on the apical side and is responsible for transport of ions, water and other nutrients. Colonocyte cytoplasm is finely granular and acidophilic, and an elongated nucleus is observed basally (Gawenis et al., 2007; Rifaat et al., 1983; Shamsuddin et al., 1982).

Goblet cells were named by Henle after their wine goblet shape, and are responsible for synthesizing, storing and secreting mucous granules and proteins (Henle, 1837). Mucins are glycoproteins secreted by goblet and epithelial cells and are the major component of intestinal mucus. In humans, it has been shown that their molecular structure is notably variable throughout the colon (Filipe, 1979). In pigs, the proximal colon and caecum mainly secrete neutral mucins, with an increasing proportion of acidic and sulfated mucins secreted in the distal part of the hindgut (Brunsgaard, 1997). Mucus aids the movement of colon contents and acts as a protective layer against mechanical stress and chemical damage to the mucosa, and under normal conditions it shelters the *lamina epithelialis mucosae* from microorganisms (Johansson et al., 2014). Approximately 10% of the cells comprising the *lamina epithelialis mucosae* are goblet cells, however there is great variability among colonic regions and individuals (Karam, 1999). In histological preparations, goblet cell nuclei are basal, more densely stained than absorptive epithelial cells and the cells are frequently deformed by a remarkably developed Golgi apparatus and granule filled cytoplasm (mucins reservoirs).

Enteroendocrine cells are located between the epithelial cells and the basement membrane and secrete hormones into the *lamina propria mucosae*. These cells are small and pyramid-shaped with granule-filled cytoplasm, usually displaying a polarity opposite to that of the columnar epithelium (Eurell and F., 2013). There are 15 subtypes of enteroendocrine cells, defined based

on their morphology and specific hormones expressed. Enteroendocrine cells account for approximately 1% of the total epithelial cell population (Schonhoff et al., 2004).

Paneth cells are secretory cells found at the crypt base (see below). Their pyramid-like shape and basal polarity are similar to goblet cells, but they can be differentiated by large eosinophylic cytoplasmic granules. Paneth cells have been associated with the innate immune response and microbiota modulation *in vivo* (Elphick and Mahida, 2005; Vaishnava et al., 2008). Their presence is normally limited to the cecum and proximal colon, and their detection in the distal colon may indicate metaplasia (Symonds, 1974).

The colon mucosa harbours a characteristic histological structure, the Lieberkühn crypts. They were first described by the German anatomist Johannes Nathanael Lieberkühn (1711-1756), who perfused wax into fresh human anatomic pieces in order to better see these microscopic structures (Lieberkühn, 1745). Despite some debate, there is strong evidence that colonic crypts are responsible for absorption and secretion of ions and secretion of mucus (Kockerling and Fromm, 1993). Crypts are formed by epithelial invaginations of the mucosa towards the submucosa, invading the underlying connective tissue. Sections cut perpendicular to the colon surface show a characteristic crypt organization resembling a row of test tubes under light microscopy (Figure 1.1). They are present throughout the colon, with the exception of the mucosa associated lymphoid tissue (MALT), ileocecal valve and anorectum transitions (Levine and Haggitt, 1989). The same columnar epithelial layer that forms the *laminae epithelialis mucosa*, containing goblet cells, mature and undifferentiated precursor epithelial cells, enteroendocrine and Paneth cells, also covers the crypts. Proliferative zones at the base of the

crypts are responsible for supplying the epithelium with new generations of specialized epithelial cells. Stem cell replication leads to transit-amplifying cells, which divide 4-5 times to reach their most differentiated and specialized cell type. These cells slowly progress from the crypt base to the top where they are unable to divide, becoming functionally mature until eventually exfoliating into the lumen due to mechanical stress and cellular detachment mechanisms (Bandaletova et al., 2002; Gordon and Hermiston, 1994). In a murine model, cells with proliferative capacity occupied ¾ of the total crypt area and the epithelial cell generation time was 39 hours (Lipkin and Deschner, 1968).

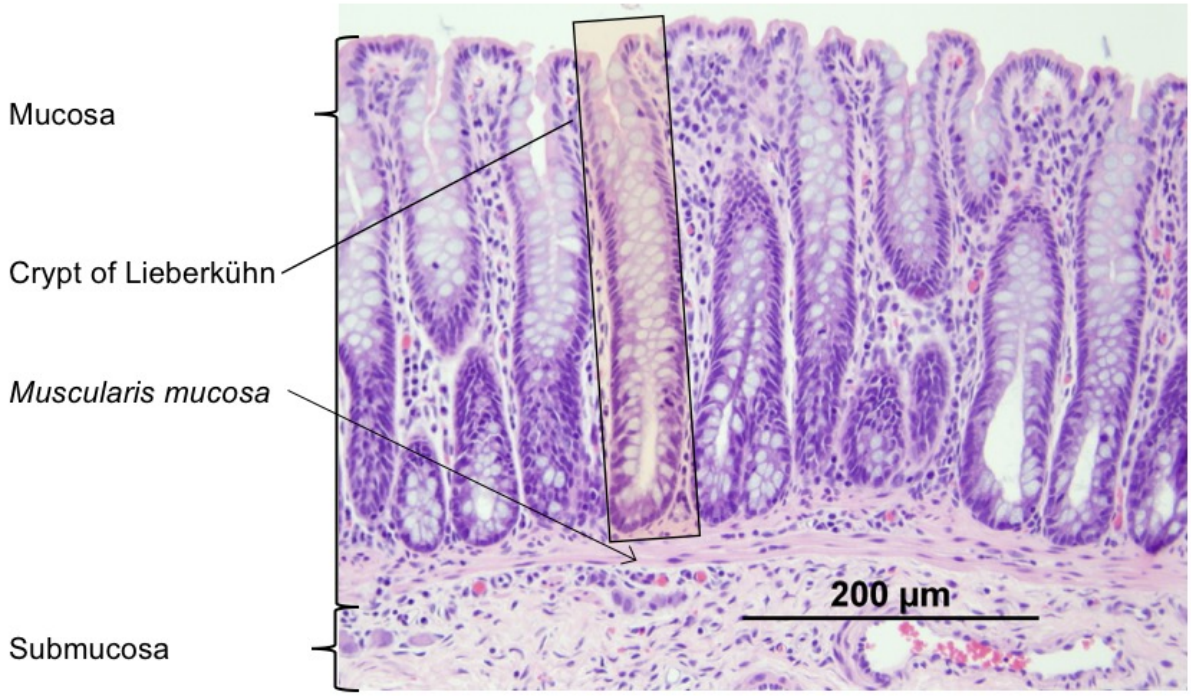


Figure 1.1 – Normal porcine colonic histology evidencing the "row of test tubes" architecture of the mucosa.

The most external cells are columnar epithelial cells, organized into crypts that together with the lamina propria and the *muscularis mucosa* form the mucosa. Immediately under is observed the submucosa.

1.2.4.2 Basement membrane, lamina propria and submucosa

Mucosa cells are supported by a thin basement membrane that consists of connective tissue invisible by light microscopy. The basement membrane is permeable to most molecules and provides anchorage for the superficial epithelial layer (Gledhill and Cole, 1984). The colon *lamina propria* is the stroma directly adjacent to the basement membrane and harbours a wide variety of cell types arranged between strands of collagen. These cells are responsible for local immune responses against luminal stimuli and consist of neuroendocrine cells, eosinophils, muciphages (macrophages containing mucus from goblet cells), mast cells and lymphocytes. The predominant cell type observed in the *lamina propria* is the plasma cell (B lymphocyte). Easily identifiable by their cartwheel shape, plasma cells are responsible for secreting immunogobulins of the IgA class, as well as IgG, IgM and IgE (Junqueira and Carneiro, 2005). IgA and IgM can be translocated across the epithelial layer to the lumen, and high levels can be detected in the mucus layer firmly attached to the epithelium (Brandtzaeg et al., 1987). T cells are found in the *lamina propria*, as well as in the colonic epithelium and the submucosa (Bartnik et al., 1980; Bull and Bookman, 1977). The majority of these cells are regulatory T cells (Treg), which are associated with the intestinal mechanisms of microbiota tolerance. Effector T cells and natural killer (NK) cells are also present and have a defensive function as they are capable of diapedesing from the *lamina propria* through the epithelium into the lumen (Hogan et al., 1985). Lymphocytes may also be found in MALT. These lymphoid follicles are distributed along the colon length within the *lamina propria*, submucosa and sometimes extending across the *muscularis mucosa*. Surface epithelium covering MALT displays cuboidal epithelial cells,

heavily infiltrated by lymphocytes. M cells, responsible for sampling luminal contents and presenting it to the underlying lymphoid tissue, are part of the surface epithelium in these areas and are difficult to recognize by light microscopy (Junqueira and Carneiro, 2005). The *lamina propria* is populated by capillary vessels and lymphatic vessels, but the latter are limited to the region immediately above the *muscularis mucosa* (Fenoglio et al., 1973).

The muscularis mucosa is a thin layer of smooth muscle cells that separates the mucosa from the deeper submucosa. The movement generated by this layer is responsible for providing an agitation-like motion to the crypt, increasing contact between the epithelium and luminal contents and aiding in expelling mucus from crypts (Junqueira and Carneiro, 2005).

Directly under the muscularis mucosa is the submucosa, formed by conjunctive tissue, blood and lymphatic vessels, lymphoid and nervous tissue. Cellular constituents are similar to the lamina propria, including lymphocytes, fibroblasts and macrophages. Importantly, the submucosa harbors two neural complexes: the plexus of Meissner and plexus of Henle (or deep submucosal plexus)(Levine and Haggitt, 1989). These plexi are composed of sensorial neurons from neural terminations near the epithelial layer (chemoreceptors) and the muscularis mucosa (mechanoreceptors), as well as effector neurons that innervate the muscular layers and enteroendocrine cells (Junqueira and Carneiro, 2005).

1.2.4.3 Muscularis propria and serosa

The most external muscle layer of the large intestine, the muscularis propria, includes fibers organized both circularly and longitudinally that are responsible for the characteristic intestinal peristaltic movement patterns (Fraser et al., 1981). Histologically similar to the submucosal Meissner plexus, Auerbach's plexus is found between the muscle layers and is the neural component of the muscularis propria (Junqueira and Carneiro, 2005; Levine and Haggitt, 1989). Numerous blood and lymphatic vessels are also present in this layer of the large intestinal wall. The muscle cell layer is wrapped in a sheath of connective tissue, the subserosal tissue. Finally, the outermost stratum of the large intestine is characterized by a layer of simple squamous epithelium (also called pavement epithelium) that is supported by the subserosal loose connective tissue. The serosa coats the entire length of the large intestine and when found covering organs is called visceral peritoneum, being a continuation of the mesentery (Junqueira and Carneiro, 2005).

1.3 The colon mucosal barrier

1.3.1 The mucus layer

The large intestine hosts an abundant and highly complex commensal microbiota, ranging between 10^{11} and 10^{14} bacteria per gram of contents, which present a range of antigens that continuously challenge host defensive mechanisms (Xu and Gordon, 2003). The mucus layer and its components are the first line of defense present in the colon, preventing microorganisms from

directly contacting the mucosa. Decreased mucin production can lead to major impairments of colonic function, as observed in clinical cases of cystic fibrosis and spontaneous colitis in mucin-specific knockout mice (Borowitz et al., 2005; Heazlewood et al., 2008). Colonic mucus is divided into two layers: one firmly attached to the epithelial cells, devoid of bacteria, and an external layer, movable and with an expanded volume due to endogenous proteolytic activity. This outer layer provides a microhabitat for the intestinal microbiota (Johansson et al., 2008). Previous studies in a rodent model have shown that the colonic mucus layer is composed of a variety of proteins, but the core molecules that build the mucus skeleton are mucins.

The mucin (MUC) gene family has 13 members, ten of which are expressed by cells within the gastrointestinal tract (Montagne et al., 2004). The intestines express mostly MUC2, while MUC5A is a secondary molecule present in the macromolecule skeleton (Johansson et al., 2008; Johansson et al., 2009). The MUC2 gene encodes a protein containing 5,200 amino acids, characterized by two central domains rich in proline (P), threonine (T) and serine (S) (PTS domains)(Gum et al., 1994; Johansson et al., 2011). MUC2 proteins interact to form a vast net-like polymer. Subunits are linked by dimeric disulphide bonds between C-termini and trimeric bonds between N-termini (Asker et al., 1998; Godl et al., 2002). It is noteworthy that swine colonic MUC2 is remarkably similar in primary structure to its human homolog, however the swine MUC2 is more heavily glycosylated, forming nets with higher density, resulting in more viscous mucus (Marshall and Allen, 1978; Podolsky and Isselbacher, 1983). The colonic microbiota is capable of using monosaccharides released from mucin metabolism as an energy source, in addition to luminal carbohydrates (Kim and Ho, 2010). Besides its selective diffusion properties, and its role in mechanical and chemical protection of the mucosa, the mucus layer is

also an important immunological tool. The array of molecules created by mucins and water is also a vehicle for secreted proteins with antimicrobial properties. Antimicrobial peptides (AMP) expressed by mammals are divided into two main families, defensins and cathelicidins, which provide protection against bacteria, virus, parasites and fungi (Jenssen et al., 2006). These are broadly present in the intestinal mucus, with more than 12 different AMPs described within the mucus in association with IgA and IgM (Hansson and Johansson, 2010; Johansson et al., 2009). IgA and IgM are secreted in parallel with mucus and are reversibly bound to mucins, facilitating the interaction of immunoglobulins with potential threats within the mucus (Antoni et al., 2013).

1.3.2 Apical junction complex

Underlying the mucus layer is the *lamina epithelialis mucosae*. Epithelial cell plasma membranes form the next level of the mucosal barrier, thanks to their impermeability to most hydrophilic solutes in the absence of specific transporters. Epithelial cells also provide a seal for the paracellular pathway in order to guarantee the efficacy of the mucosal barrier. The first scientific report of a mechanism to keep epithelial cells together, sealing the paracellular space, described a "terminal bar" featured by all epithelia, possibly playing a role in segregation of fluids with different compositions (Bonnet, 1895). The terminal bar is now referred to as the apical junction complex, and it is composed of tight junctions, the most apical structure, followed by adherens junctions, desmosomes and gap junctions.

Tight junctions are supported by a ring of actin and myosin filaments that terminate at the plasma membrane, aiding in regulation of the barrier. These filaments are in turn bound to membrane

scaffolding proteins (claudins and occludins). Zonula occludens 1 and 2 (ZO-1 and ZO-2) are crucial to tight junction assembly and maintenance due to their multiple domains for interaction with other proteins. These domains are capable of interacting with tight junction regulatory proteins including actins (Ma and Anderson, 2006). Basal to the tight junctions are the adherens junctions, characterized by the presence of the intercellular protein cadherin and its intracellular adhesion molecule, β-catenin. Adherns junction proteins are also connected to the ring of perijunctional actin filaments (Turner, 2009). Next are desmosomes, transmembrane proteins analogous to cadherin, but associated with intermediate filaments rather than actin. Desmosomes confer protection against mechanical damage to the epithelium due to luminal flow and peristaltic movements (Ma and Anderson, 2006). Gap junctions are responsible for permitting the passage of small molecules through the paracellular pathway, coordinating epithelial functions of secretion and exocytosis (Leite et al., 2002).

The contribution of the apical junction complex to the mucosal barrier is remarkable, isolating luminal from basolateral contents to such a degree that an electrical resistance can be measured between the two compartments. Transepithelial electrical resistance (TER) is a measurement of the magnitude of the epithelial barrier function based on the ability of the epithelium to separate ionic charges. Based their electrical resistance, epithelia may be classified as “tight” or “leaky”, with tighter tissue having higher resistance and leakier tissue with relatively lower resistance (Powell, 1981). The colon is classified as moderately leaky, with the ability to allow large amounts of isosmotic fluids to cross its tissue. A gradient of electrical resistance is observed along the apical/basal axis of the crypts, with apical/surface region being less leaky (Ma and Anderson, 2006).

1.3.3 Inflammatory modulation of the mucosal barrier

Cytokines are known to produce a range of effects while modulating the colonic inflammatory response. The mucosal barrier is a target for these signalling molecules, as they are capable of modulating the apical junction complex in order to respond to inflammatory stimuli. Madara & Stafford were the first to investigate the effects of cytokines, particularly interferon gamma (IFN-γ), on the intestinal epithelium (Madara and Stafford, 1989). These authors observed that exposure of colonic epithelial cells to IFN-γ was responsible for a delayed increase in paracellular permeability, perceived at 72 hours after exposure, instead of an acute response. This effect was attributed to a decrease in synthesis and increase in degradation of the tight junction protein ZO-1 (Adams et al., 1993). Physiological concentrations of the pro-inflamatory cytokine TNF-α (tumor necrosis factor-alpha) have also been associated with increased mucosal permeability *in vivo* and *in vitro*. TNF-α effects have been associated with activation of a nuclear factor, nf-κb, which leads to a decrease in ZO-1 synthesis and a disturbance in the perijunctional actin rings (Bruewer et al., 2003; Gitter et al., 2000a; Gitter et al., 2000b; Ma et al., 2004).

Mucosal damage triggers the secretion and activation of a wide range of cytokines and inflammatory mediators. There is a general consensus that pro-inflammatory molecules such as TNF-α, IFN-γ, interleukin-1β (IL-1β), IL-4, IL-6, IL-12, IL-13, insulin, insulin-like growth factor, and hepatocyte growth factor lead to an increase in intestinal tight junction permeability, while other non-inflammatory mediators including IL-10, TGF-β, and epidermal growth factor seem to aid in maintenance of the epithelial apical junction complex barrier function (Ma and

Anderson, 2006). Increased tight junction permeability induced by the cytokine response has been proposed as an important contributor to the intestinal inflammatory process, as it facilitates the paracelullar permeation of toxic luminal agents (Hollander, 2002; Ma et al., 2004; Madara and Stafford, 1989; Suenaert et al., 2002). Despite increased interest of the scientific community in the epithelial response to cytokines, understanding of the intracellular and molecular mechanisms associated with these effects remains incomplete.

1.4 Colon ionic transport

The colon contributes significantly to absorption and digestion of nutrients through the digestion of complex carbohydrates by the resident microbiota, uptake of nutrients resistant to prokaryotic digestion, and reabsorption of water (Christl and Scheppach, 1997). Traditionally, absorption was defined as an exclusive process of surface cells, while secretion was thought to be exclusive to crypt cells (Field et al., 1989; Welsh et al., 1982). However, more recent studies have yielded evidence that surface cells and crypt cells are both involved in absorption and secretion in the colon (Geibel et al., 2001; Ikuma et al., 1999; Kockerling and Fromm, 1993; Rajendran et al., 1999).

1.4.1 Absorption

Absorption in the colon occurs by two mechanisms: electroneutral and electrogenic ionic transport. The major driving force for these mechanisms is provided by a basolateral Na^+/K^+-

ATPase. The majority of NaCl absorption through the colon occurs *via* electroneutral pathways. A family of Na/H exchangers (NHE) has been described in the colon. NHE1 is found in the basolateral membrane, while NHE2 and NHE3 are located on the apical side (Robert et al., 2001). A Na^+/H^+ exchanger regulatory factor (NHERF) has been described in the small intestine, and has been shown to be an important part of the cAMP-mediated inhibition of sodium absorption by NHE3 in the kidney (Weinman et al., 2000; Yun et al., 1997). However, the NHERF role in colonic absorption is yet to be clarified. Anion exchangers of two types have been identified in the colon: the Cl^-/HCO_3^- system (associated with Anion Exchanger 1 protein) and the Cl^-/OH^- exchanger (down-regulated in colonic adenomas) (Rajendran et al., 2000).

Electrogenic absorption of sodium occurs in the distal colon to enhance Na^+ uptake. The Na^+ channel (ENaC) is localized in the apical membranes of colonocytes and is inhibited by amiloride (Benos et al., 1992). Electrogenic sodium uptake acts as a driving force for chloride absorption *via* the paracellular shunt. Sodium and chloride ions are transported out of the cells through Na^+/K^+-ATPase and basolateral Cl^- channels and Cl/HCO_3^-, thus producing net absorption of these ions (Greger et al., 1996).

1.4.2 Secretion

Secretion is another important role played by the colon as part of the digestive tract. It is balanced by the absorption of electrolytes in a way that homeostasis in maintained, critical for preventing pathogenic loss of electrolytes and water. It is theorized that under physiological conditions secretion by the crypts aids in the removal of mucus from the crypt lumen, enhancing

fluid secretion (Halm and Halm, 1999). Apical potassium and chloride channels actively secrete KCl, which is balanced through the basolateral uptake of Na^+, K^+ and Cl^- *via* the basolateral NKCC1 system (Dawson, 1991). It has been demonstrated that activation of the mammalian colon NKCC1 is paired with apical excretion of chloride *via* the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), enhancing net chloride secretion. This mechanism is controlled through the epithelial capacity of sensing the fall in intracellular Cl^- and/or cell volume at the onset of secretion (Lytle and Forbush, 1996). In fact, CFTR has been described as an important source of apical chloride secretion in the colon due to its high expression under physiological and pathological conditions (Chao et al., 1994; Greger, 2000; Greger et al., 2001). In addition, Calcium-activated Chloride channels (CaCC) have been found in human colonic carcinoma and T84 cells (Cliff and Frizzell, 1990; Worrell et al., 1989). As the name suggests, CaCC is activated by increasing levels of intracellular calcium and leaks Cl^- into the lumen. To maintain ionic balance, apical and basolateral potassium channels are also present. In addition, basolateral K^+ channels are important for maintaining membrane hyperpolarization, acting as a driving force for chloride secretion and sodium absorption (Budinger et al., 1986; Butterfield et al., 1997; Loo and Kaunitz, 1989).

Net transport of water in the human colon is estimated to be up to 1.5 liters per day (Said and Seetharam, 2006). Osmotic forces generated by absorption and secretion of ions and larger molecules drive this fluid movement. The extent to which this movement relies on the paracellular pathway or on cellular transport remains unknown. The colon is known to harbour channels specific for water transport called aquaporins. Throughout the gastrointestinal tract, at least seven different aquaporins have been described, with AQP3, AQP4 and AQP8 being

expressed by the colonic mucosa (Koyama et al., 1999; Thiagarajah and Verkman, 2006). However, aquaporins do not seem to be essential for colonic fluid absorption and fecal dehydration, as shown in a knock-out mouse model (Wang et al., 2000). Results of this work indicate that either another pathway plays a major role, or there are additional aquaporins yet to be described. In addition, Hasegawa and collaborators have shown that CFTR is able to accommodate dehydrated chloride anions as well as small solutes and water, behaving as another pathway for water movement (Hasegawa et al., 1992).

The colonic epithelium is also responsible for secreting large amounts of mucus, required for mechanical and immunological protection of the mucosa. Mucins, the main molecules that form mucus, are secreted by goblet and crypt columnar epithelial cells *via* exocytosis stimulated by increased intracellular levels of cAMP or Ca^{2+} (Halm et al., 1995; Johansson et al., 2011; Neutra et al., 1982; Specian and Neutra, 1982). Release of preformed mucus is stimulated by cholinergic neurons, while increase in intracellular cAMP induces the release of preformed mucus and *de novo* synthesis of it. Epithelial cells require the presence of prostaglandins for releasing mucus filled vesicles into the luminal canal (Forstner, 1995; Jarry et al., 1994a; Jarry et al., 1994b).

1.5 Pathophysiology of colonic diarrhea

The word diarrhea derives from the Greek *diarhein*, formed by the radical *dia*, meaning through and the radical *rhein*, meaning flow. Diarrhea compromises intestinal pathological processes resulting in increased volume and frequency of defecation (Klein, 2013). Fecal loss of electrolytes and water may achieve high amounts and become life threatening, especially in risk

groups such as nursing and weanling individuals. It is imperative to understand the disturbance of physiological mechanisms that lead to clinical disease in order to understand and prevent lethal cases of diarrhea. The main mechanisms of diarrhea are osmosis, active secretion, exudation and abnormal motility. It is not uncommon to find two or more of these forces involved in diarrheal illnesses.

1.5.1 Osmotic diarrhea

Ingesta containing poorly absorbable aqueous solutes are osmotically active. Their presence in the lumen restrains normal water and electrolyte absorption, driving water from extracellular fluids and impairing reabsorption of water from luminal contents. Individuals with normal gut function are susceptible to developing osmotic diarrhea when large amounts of poorly absorbable solutes such as sorbitol or Mg^{2+} are ingested. Osmotic diarrhea may also develop when an individual with absorptive defect ingests normally absorbable nutrients, as in the case of pancreatic insufficiency. Maldigestion and overload of undigested carbohydrates and lipids reaching the colon are promptly metabolised by the microbiota *in situ*, overwhelming the colonic absorption capacity and culminating in osmotic diarrhea. This type of diarrhea ceases after fasting (Klein, 2013; Woods, 1990).

1.5.2 Secretory diarrhea

Intestinal secretion and absorption are continuous processes and under physiological circumstances there is net absorption. Overstimulation of intestinal secretory function usually leads to large volumes of feces in the absence of leukocytes or erythrocytes in the stool, an absence of fever and a lack of osmotic anion gap in the stool (Said and Seetharam, 2006; Woods, 1990). The osmotic gap is defined as $OG = 290 – 2([Na^+] + [K^+])$, where 290 mM is the assumed osmolarity of blood plasma and OG > 50 mM is considered normal. Normal stools have low sodium and high potassium concentrations due to colonic absorptive capacity for sodium. Chloride concentration is low and bicarbonate levels are similar to plasma (Erickson et al., 2015; Klein, 2013). Second messengers associated with the increased secretion into the intestinal lumen are cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), calcium (Ca^{2+}) and nitric oxide (NO). A number of agents can stimulate secretion, including bacterial enterotoxins, hormones, fatty acids and inflammatory mediators. Cholera, cause by the toxin produced by *Vibrio cholerae,* is a classic model of secretory diarrhea. The toxin increases intracellular cAMP, stimulating anion secretion, resulting in loss of water and diarrhea (Peterson and Ochoa, 1989). Neurohormonal substances and toxins, such as those produced by *Helicobacter pylori* and *Cryptosporidium* spp. are able to increase intracellular calcium, thus activating CaCC and increasing chloride secretion (Guarino et al., 1998; Guarino et al., 1994).

1.5.3 Exudative diarrhea

Excessive colonic inflammatory response leads to a disruption of the mucosal apical junction complex, affecting mucosal barrier function and generating large quantities of inflammatory exudate. Additionally, the hydrostatic pressure in blood vessels and lymphatics will push water, electrolytes, protein and even blood cells through the mucosa. Molecules that physiologically would not be observed within the lumen will leak into it, which may result in hypoproteinemia in chronic cases (Erickson et al., 2015; Ettinger and Feldman, 2009).

1.5.4 Diarrhea due to abnormal bowel motility

Abnormal peristalsis may affect the normal flux of the ingesta in different ways. Significant increases in peristaltic movements in the small intestine may deliver excessive amounts of chyme to the colon, exceeding its absorptive capacities, resulting in diarrhea (Klein, 2013; Read et al., 1980). Abnormalities in colonic motility such that luminal contents are emptied before adequate metabolism can occur is associated with diarrhea in patients with irritable bowel disease (Drossman et al., 1977).

1.6 Infectious agents associated with swine colonic diarrhea

1.6.1 Epidemiological aspects

Sus scofra domestica was domesticated approximately 9,000 years ago (Giuffra et al., 2000). Pigs are a valuable source of protein and fat that were historically farmed as a way of disposing of municipal garbage on a large scale (Morse, 1908). In modern society pigs commercially raised are protected by biosafety practices designed to prevent infectious agents from coming into contact with the herd. However, deficiencies are always present and many hazards still surround pork production. Infectious diseases are particularly important due to their often rapid and potentially disastrous outcomes.

Enteric diseases affecting pigs are unquestionably a major problem in swine production. Pigs of different ages are exposed to a range of infectious agents capable of disrupting colonic homeostasis. In general, unweaned piglets may have colonic lesions due to Rotavirus, *Clostridium difficile*, *Brachyspira hyodysenteriae, Salmonella*, or *Toxoplasma* sp.. Recently weaned pigs may be affected by *Salmonella, Oesophagostomum* spp. and tricothecene toxin (*Fusarium* spp.). Growers and finishers may be infected by all of the previous, as well as *Lawsonia intracellularis*, *Brachyspira* spp., *Trichuris suis*, African swine fever virus and *E. coli* 0139:K12:H1 (edema disease) (Straw et al., 2006). However, application of modern biosecurity measures has led to the control of several of these diseases.

Petersen and colleagues surveyed 154,347 finisher pigs from 90 different Danish herds before slaughter and found that 0.25% of the animals had clinical signs of diarrhea (Petersen et al.,

2008). However, diarrhea was only accounted for when a pig was observed defecating loose stools, excluding for example diarrheic feces on the floor. Christensen and others reported diarrhea as the second most common reason for antibiotic treatment of Danish pigs, accounting for 23% of all treatments (Christensen et al., 1994). A British report found that 50.5% of 105 herds had experienced a scour problem within the previous three years, and colitis was associated with 44.3% of cases (Pearce, 1999). A study carried out between 1997 and 1999 in 98 pig units in the United Kingdom focused in elucidating the causes of colitis and typhlocolitis (Thomson et al., 2001). *Brachyspira pilosicoli* was the most common primary agent, found in 18% of the cases, followed by *B. hyodysenteriae* (13%), *Lawsonia intreacellularis* (10%), *Salmonella* spp. (6%) and *Yersinia* spp. (4%). Noteworthy, unclassified *Brachyspira* spp. were detected in 12% of the colitis cases.

Despite the economic importance of infectious colonic diarrhea there is a major gap regarding the North American scenario. Major epidemiologic surveys on the prevalence of the different etiologic agents of diarrhea in pigs, including *Brachyspira* spp. are absent in the current scientific literature. A study focused at *L. intracellularis* found seropositive pigs in the three investigated provinces: Alberta, Quebec and Ontario. The authors also suggested that farrow-to-finisher farms are more likely to have seropositive pigs than finishing only and multisite farms (Paradis et al., 2007).

1.6.2 Pathogenesis of haemorrhagic colitis due to non-spirochaetal bacterial infection

Understanding the means employed by pathogens that result in host lesions is essential in veterinary and human medicine. A current challenge to veterinarians is to find better prophylactic tools to prevent infectious diarrhea. Understanding the mechanisms employed by bacteria to overcome host defenses and trigger diarrhea is a key point in this quest. It allows the development of new prophylactic tools and better usage of the existing ones. Different pathogens may share strategies to invade and proliferate within the intestines, and comparing the pathogenesis of diseases may be of great guidance during the process of elucidating the pathogenesis of emergent diseases. This was the case with *E.coli* associated diarrhea, which was clarified based on comparisons with *Vibrio coli* (Pierce, 1973; ter Huurne and Gaastra, 1995).

Contemporary biosafety measures have greatly contributed to the control of several of the above cited agents of colonic diarrhea in pigs. Currently differentials for haemorrhagic diarrhea in grower pigs from commercial farms include *Salmonella* enterocolitis and *Lawsonia intracellularis* colitis. Lesions associated with these diseases are not restricted to the colon and may affect the small intestines as well. The pathogenesis mechanisms used by these pathogens have been at least partially characterized and are apparently quite distinct.

S. cholerasuis and *S.* typhimurium are the most common *Salmonella* isolated from pigs. Both are capable of causing enterocolitis, while *S.* cholerasuis also causes systemic disease. Briefly, gastrointestinal findings are yellow watery scours sporadically permeated by blood. Gross lesions are characterized by diffuse necrotic enteritis or typhlocolitis (Levine et al., 1945; Reed et al., 1986). Clinical and pathological characteristics of *Salmonella* infection are highly variable,

mostly due to the more than 200 virulence factors characterized from the genus so far (Schwartz, 1999). Pigs that develop enterocolitis are commonly immunocompromised with concurrent debilitating processes, or exposed to very high infectious doses of the pathogen (Reed et al., 1986). It has been shown that over 10^7 cells/g of intestines are necessary for successful colonization and lesion production in the lower gastrointestinal tract (Bohnhoff et al., 1954). Upon colonization of the distal parts of the intestine, *S.* Typhimuruim employs type 1 fimbriae in order to attach to the apical membrane of enterocytes. Within two hours of oral inoculation it is possible to observe morphologically intact bacteria within enterocytes, free in the cytoplasm or attached to the cell membrane, and mesenteric lymph nodes. The pathogen is also capable of invading goblet cells and M cells, with serovar Cholerasuis being more frequently found within M cells than Typhimurium. The two serovars invade the intestinal mucosa through distinct routes, with Cholerasuis being more commonly associated with colonic lesions and ileal Peyer's patches, and Typhimurium showing no specific tropism for any intestinal region (Meyerholz and Stabel, 2003; Reed et al., 1986; Schauser et al., 2004). Before invading the tissue the pathogen induces mucosal exfoliation due to caspase 3-dependent and independent epithelial cell death (Schauser et al., 2005). Invasion of the epithelium triggers the production of several cytokines by the epithelium and macrophages such as IL-8. As a result of IL-8 production and release into the lamina propria, neutrophils from the circulation and submucosa are recruited through SP-1 effector SipA protein (McCormick et al., 1995; Skjolaas et al., 2006; Volf et al., 2007; Wall et al., 2007). The tissue damage caused by the immune response elicited during invasion by *Salmonella* plays a role in the onset of diarrhea and is associated with the intracellular release of SopB by the pathogen. This protein has inositol phosphate phosphatase activity, increasing

intracellular concentrations of inositol 1,4,5,6-tetrakisphosphate, a molecule that indirectly increases chloride secretion by antagonizing the inhibition of CaCC-dependent secretion (Norris et al., 1998).

Proliferative enteropathy (PE) is associated with infection of the intestines by the Gram negative, vibroid shaped *Lawsonia intracellularis.* PE is characterized by thickening of the intestinal mucosa due to adenomatous proliferation of immature epithelial cells (McOrist et al., 1995a). Initially thought to be restricted to the ileal region, the pathogen was later also associated with colitis as reported by Jensen and collaborators (Jensen et al., 2006). Lesions observed ranged from thickened colonic mucosa without complications to extensive necrotizing colitis. Excessive mucus was not observed but haemorrhagic colitis was reported. Although a small portion of the cases (4%) had exclusively colonic lesions, 18% of these were diagnosed with enterocolitis. PE pathogenesis still remains mostly unclear, as most of the virulence factors are not well characterized. The major mechanism of cellular invasion employed by the pathogen is induction of enterocyte hyperplasia after attachment and entry through the apical surface. Currently, no specific adhesins have been reported and entry into the cell appears to require specific bacterium-host cell interaction (Lawson et al., 1995; McOrist et al., 1995b). Bacteria enter the cell *via* an entry vacuole, and following escape from the vacuole the organisms can be observed free in the cytoplasm. This behaviour is similar to other pathogens such as *Shigella, Listeria* and *Rickettsia* spp. Interestingly, these three genera are capable of producing lytic toxins that facilitate escape from the entry vacuole. *L. intracellularis* has been reported to produce a hemolysin, which is suggested to play a similar role in pathogen escape from the vacuole (McCluskey et al., 2002). Once free inside immature epithelial cells, the bacteria replicate and stimulate cellular mitosis,

transmitting the organisms to daughter cells. This cycle is responsible for generating the characteristic thickened mucosal layers that may progress to necrosis, leading to rapid death of the host. Chronically affected individuals that survive the acute phase of the disease usually also display thickening of the muscularis mucosa (McCluskey et al., 2002; Ward and Winkelman, 1990). Many aspects of the pathogenesis of PE diarrhea remain unclear. Most of the efforts directed to control of the disease focus on pathogen eradication, but better understanding of the pathophysiology of the disease could lead to development of interventions that improve survival rates and minimize lesions.

1.7 Swine associated *Brachyspira* spp.

Intestinal spirochaetes of the Genus *Brachyspira* are inhabitants of the indigenous microbiota of healthy pigs (Patterson et al., 2013; Stege et al., 2001). This genus belongs to the domain Bacteria, phylum *Spirochaetes*, class *Spirochaetes*, order *Spirochaetales*, family *Brachyspiraceae* (Stanton, 2006). It compromises seven formally recognized species, harboured by different hosts ranging from humans to pigs and chickens (Table 1.1). Several other taxa have been recognized within *Brachyspira,* but these "species" currently lack formal standing in nomenclature.

Table 1.1. Summary of recognized *Brachyspira* species and their habitat.

Taxon	Pathogenicity to pigs	Main recognized hosts
Brachyspira hyodysenteriae	Pathogenic	Pig
Brachyspira intermedia	Questionably pathogenic	Pig, poultry
Brachyspira innocens	Non-pathogenic	Pig
Brachyspira murdochii	Pathogenic	Pig, rat
Brachyspira pilosicoli	Pathogenic	Pig, poultry, dog, human, horse
Brachyspira aalborgi	Non-pathogenic	Human
Brachyspira alvinipulli	Non-pathogenic	Poultry

Brachyspira spp. are oxygen tolerant, Gram negative cells with motility. Periplasmic flagella surrounding the protoplasmic cylinder and covered by an outer envelope confer this ability to the spirochaetes. Acquired resistance to antibiotics, such as rifampicin, colistin, vancomycin and spectinomycin is often observed among species of this genus (Brooke et al., 2003; Holt, 1978; Jenkinson and Wingar, 1981).

Six *Brachyspira* spp. are known to colonize pigs of different ages, from post weaning to finisher pigs. Weakly haemolytic *B. innocens* is considered non-virulent to pigs, but is often associated with other pathogenic *Brachyspira* species in clinical cases (Moxley and Duhamel, 1999; Straw et al., 2006). Species with demonstrated pathogenicity in pigs are *B. pilosicoli, B. murdochii, "B. suanatina", B. intermedia* and *B. hyodysenteriae* (Jensen et al., 2010; Råsbäck et al., 2007a; Taylor and Alexander, 1971; Taylor et al., 1980). *B. pilosicoli* infection in pigs causes spirochaetal colitis, characterized by a mild diarrhea, with "wet cement" to watery feces, sometimes progressing to mucoid (Trott et al., 1996). *B. murdochii* has been associated with watery diarrhea and colitis, both in experimentally infected and naturally infected pigs (Jensen et al., 2010; Palzer et al., 2008). "*B. suanatina"* was isolated from Swedish and Danish pigs with diarrhea, as well as wild-living mallards (Råsbäck et al., 2007a). Authors have reported that *"B. suanatina"* isolated from mallards is capable of inducing mucohaemorrhagic diarrhea in recently weaned pigs. Initially considered non-pathogenic to pigs, *B. intermedia* was described in 1997 (Stanton et al., 1997). However, there are reports of strains of *B. intermedia* associated with diarrhea in pigs (Binek and Szynkiewicz, 1984; Burrough et al., 2012b; Fellstrom and Gunnarsson, 1995). The pathogenic status of this species remains unclear, complicated by the

amazing amount of diversity observed among isolates identified as *B. intermedia* (Phillips et al., 2010).

The most economically significant and clinically severe disease associated with spirochaetes in pigs is caused by *B. hyodysenteriae* (Taylor and Alexander, 1971). The syndrome is named swine dysentery (SD) and is economically the most important malady restricted to the large intestine of pigs, affecting 10-33% of piggeries around the world (Bellgard et al., 2009; Hampson et al., 2000; La and Hampson, 2001; Moxley and Duhamel, 1999; Song et al., 2012). Transmission of the agent occurs *via* the fecal-oral route. SD is characterized by fibrino-necrotic typhlocolitis and mucoid to blood stained diarrhea. Although SD was first described in 1921 (Whiting et al., 1921b), it was not until 1971 that *B. hyodysenteriae* (at the time, *Treponema hyodysenteriae*) was identified as the agent responsible for SD (Glock, 1971; Glock et al., 1974; Harris et al., 1972).

Currently, *B. hyodysenteriae* is found in all major pig-producing countries. Its economic importance is due to an increase in feed conversion ratio, mortality, medication and eradication procedures, all leading to greater production costs (Harris and Glock, 1971). Estimates of the costs associated with the disease are on the order of 10% of total paid value per 100 kg of pork (average weight at slaughter) (Dufresne, 1999). In addition, abattoirs usually apply penalties to affected carcasses due to size variation.

1.7.1 Epidemiology

Historically, grow-finish farms acquired weaned piglets through auction marts and assembly yards, where lack of hygiene and biosecurity in general would facilitate the spread of pathogenic bacteria and other parasites. Pathogenic *Brachyspira* are most likely to enter a healthy herd by introduction of a chronically affected individual from an infected farm. Carrier individuals could also be a source of infection. Even though it has been known since the 1980s that pigs can shed *B. hyodysenteriae* for up to 80 days, introduction of new, potential carrier pigs to farms in the last century was done in an improper manner, without quarantine or scanning for pathogens (Fisher and Olander, 1981). Transmission on and between farms is enhanced by the pathogen's ability to survive in faeces for 48 days in temperatures between 0 and 10°C (Chia and Taylor, 1978).

Essentially, pigs of any age are susceptible to pathogenic *Brachyspira* spp., but fattening herds are more commonly affected than breeding herds. Once in the barn, morbidity due to SD can reach 90% and mortality may reach over 50% of the herd. Chronically affected herds tend to have grower-finisher pigs developing SD in a cyclic manner, with 30% of the herd affected (Harris and Glock, 1971; Joens et al., 1979). The most critical predisposing factors that lead to this pattern of disease by *B. hyodysenteriae* remain unknown.

Pigs, however, are not the only source of introduction of pathogenic *Brachyspira* onto farms. Contaminated clothes, boots, trucks and other fomites can harbour infectious spirochaetes and deliver them to pigs. Wild rodents have been shown to be reservoirs and their potential of spreading pathogenic *B. hyodysenteriae* to pigs has been demonstrated (Hampson et al., 1991).

In addition, intestinal spirochaetes are commonly isolated from birds, more specifically migratory and water-living species, which intensifies their potential to spread the bacteria in the environment (Jansson et al., 2004; Jensen et al., 1996; Råsbäck et al., 2007a; Rubin et al., 2013b).

1.7.2 Diagnosis

Observation of clinical signs is key towards a final diagnosis of *Brachyspira* infection. *Brachyspira* associated diarrhea usually starts as soft feces, which may progress to watery, mucoid and bloody in the case of *B. hyodysenteriae* infection. However, these stages are not observed in every individual, and not all infected pigs will have bloody diarrhea. When any degree of diarrhea is observed, it is important to rule out other possible pathogens such as *Salmonella*, *L. intracellularis, Clostridium perfringens* type C, *Isospora suis*, *Eimeria* spp., and *Trichuris suis*. Necropsy is often performed by veterinarians for observation of lesions and other findings in internal organs. It also allows for sample collection and submission to the diagnostic laboratory. Macroscopically, affected individuals have lesions limited to cecum and colon. Those may be focal or diffuse, acute or in process of chronification. The colonic lumen contains highly viscous mucus, with fibrin and possibly bloodstains. Chronic cases show superficial necrosis, with feed and fibrin adhered to erosion sites (Glock et al., 1974; Hampson et al., 2006; Harris et al., 1972; Taylor and Alexander, 1971; Whiting et al., 1921a). However, none of these signs is pathognomonic for the disease.

Laboratory diagnosis of *Brachyspira* associated diarrhea begins at the farm. Proper sampling is imperative for a trustworthy final diagnostic. Clean collecting apparatus as well as fresh samples greatly contribute to successful diagnosis. Samples improperly shipped to the laboratory may not be suitable for culture in selective media, which leaves no other option than the use of molecular tools. Non-optimal samples for culturing are usually frozen or kept on temperatures greater than 5°C for extended periods of time or come from treated pigs. All these factors lead to spirochaete death (Harding et al., 2013).

Traditional culture and biochemical tests still remain important to diagnosis and characterization of *Brachyspira* spp. as they provide material for further identification and have low detection limits associated with high sensitivity and specificity (Patterson et al., 2013). Growth on selective media agar usually takes 2-4 days at 42°C. *Brachyspira* grows under anaerobic conditions, but it has been shown that small amounts of oxygen contribute to bacterial growth (Stanton and Lebo, 1988). *Brachyspira* does not form visible colonies on solid media, but creates zones of haemolysis that allows for growth verification. Species identification of cultured isolates can be based on biochemical tests such as indole production, α-galactosidase and β-glucosidase activity and hippurate hydrolysis. A classification scheme was proposed for *Brachyspira* species based on these biochemical traits (Fellstrom et al., 1999). However, these characteristics can be variable among clinical isolates. At this point it is impossible to speciate *Brachyspira* isolates solely based on culture and phenotypic features. This fact, coupled with the challenges of selective culture of these fastidious organisms has led to an increasing reliance on molecular diagnostic techniques.

The use of PCR for detection and identification of *Brachyspira* from clinical cases was an important addition to diagnostic laboratories, given the short time necessary for results when compared to culture. Multiple PCR protocols are available in the literature for detection of *B. hyodysenteriae* and *B. pilosicoli* (La et al., 2006; La et al., 2003; Nathues et al., 2007). Identification of pathogenic *Brachyspira* isolates beyond the genus level is often a challenge. Many authors have reported field cases where mucoid or mucohaemorrhagic diarrhea was associated with *Brachyspira*, but failed to speciate the isolates, calling them "atypical" (Fellstrom et al., 1999; Hommez et al., 1998; Thomson et al., 2001). Previous attempts focused at using the 16S rRNA gene for identification of isolates, but the gene is conserved to a degree that gives it insufficient resolution required for species level differentiation (Pettersson et al., 1996). The vast majority of diagnostic PCR techniques for *Brachyspira* detection and identification are based on the NADH oxidase (nicotine adenine dinucleotide phosphate-oxidase, *nox*) gene (Akase et al., 2009; Rohde et al., 2002; Rubin et al., 2013a). It is present amongst species of the *Brachyspira* genus, and its genetic sequence is conserved between species in such degree that suits the needs for speciation of strains (Song and Hampson, 2009).

Reproducible serological tests for herd surveillance of SD have been described (Song et al., 2012). These are IgG based ELISAs (Enzyme-linked immunosorbent assay) and use whole-cell preparations for coating for ELISA plates. The authors suggest that these serological tests may be applied for detection of serum immunoglobulin for *Brachyspira* spp. from meat juice samples at the slaughterhouse level. Very limited data is available on the use of these assays, and further investigation of their specificity is required. In addition, the ELISA assays described by Song et

al. do not have the ability to differentiate *Brachyspira* species, and are to be used as epidemiological survey tools only.

Hematological assessment of pigs with SD has been somewhat neglected by the scientific community. Leukogram analysis shows increased numbers of leukocytes and left shift, where immature neutrophils predominate. $CD4^+$ and $CD8^+$ T cells are the main lymphocytes associated with host response to *B. hyodysenteriae* (Jonasson et al., 2004; La and Hampson, 2001). Packed-cell volumes are variable, suggesting that blood loss is not considerable. However, the onset of diarrhea may distort this as a result of dehydration. Serum protein may increase with the progression of diarrhea, as expected. In addition, electrolytes also experience significant shifts with the disease evolution. The most prominent changes observed are hyperkalemia, increased chloride and bicarbonate levels. These are evidence of acidosis, that ultimately results in death when not treated (Glock, 1971). Recently, a study of field cases revealed that IL-1β and IL-6 were found in high concentration in infected pig sera (Jacobson et al., 2011). IL-1 is related to activation of $CD4^+$ T cells, while IL-6 stimulates the production of antibodies by plasmocytes. As previously stated, many gaps still remain to be filled regarding the host response to *B. hyodysenteriae* infection.

1.7.3 Control and treatment

Currently, there are no registered vaccines to prevent or treat SD in North America. However, it is possible to control the disease through good hygiene practices, biosecurity and antibiotic therapy.

Procedures related to hygiene within the farm are critical to reduce introduction and spread of the *Brachyspira* spp. These should be applied outside the farm, while controlling visitors and vehicles that enter the premises, and preventing direct and indirect contact of pigs with other domestic and wild species. Feed and water should be also free from contamination. Previous reports have shown that waterfowl may carry *Brachyspira* as part of their indigineous microbiota, becoming a potential contamination source (Martinez-Lobo et al., 2013; Rubin et al., 2013b). When introducing new animals to a herd, the disease status of the farm of origin should be investigated so that a decision can be made regarding prophylactic treatment. Disinfection of transit pathways, use of foot dips and pools to reduce transmission from pen to pen (within herd) are also important. Rodent control, renovation of buildings and an environment free of stress also contribute to preventing SD introduction and outbreaks. When the latter occurs in a farm, the disease usually results in contamination of the premises, which means that the farm remains infected unless whole-herd depopulation followed by restocking is performed. In fact, after an outbreak, farrow-to-finish herds remain infected as sows with subclinical infection continue to shed the pathogen in their feces (Patterson et al., 2013). However, depending on the health status of the herd, diarrhea will be more or less severe and is likely to affect growing or finishing pigs due to the removal of medication used to prevent respiratory infections, favouring the appearance of clinical SD at this stage (Alvarez-Ordonez et al., 2013).

Treatment of SD relies on the use of antimicrobial agents such as pleuromutilins, macrolides and lincosamides (e.g. tiamulin, valnemulin, tylosin and lincomycin) (Novotna and Skardova, 2002). However, not all drugs are permitted due to country-specific legislation, e.g. nitroimidazole drugs are not licensed for use in pigs in the European Union and USA. Typically, tiamulin is the

antibiotic of choice. Routes of administration include intramuscular injection once a day for three days or oral via drinking water for five days. Continuous treatment of herds with antibiotics for extended periods (sometimes as growth promoters) has been banned from many countries, due to uncontrolled use of such drugs resulting in decreased susceptibility of *Brachyspira hyodysenteriae* isolates to tiamulin (Hidalgo et al., 2011).

1.7.4 Pathogenesis

Pathogenic *Brachyspira* colonize the large intestine and can be found on the luminal surface and within crypts. Invasion of enterocytes, goblets cells and the *lamina propria* of pigs diagnosed with SD has been reported based on electron microscopy and histopathology analysis of infected tissue (Glock et al., 1974; Taylor and Blakemore, 1971). Most of what is known about *Brachyspira*-associated diarrhea pathogenesis is related to *B. hyodysenteriae* infection. Even though it has been almost 100 years since the first scientific description of SD, the pathogenesis is yet to be fully understood. Virulence factors that may contribute to disease development are the motility of the bacteria, resistance to oxygen toxicity, chemotaxis to mucus, haemolysins and lipooligosaccarides (Kennedy and Yancey, 1996; Milner and Sellwood, 1994; Nibbelink and Wannemuehler, 1991; Rosey et al., 1996; Stanton et al., 1999).

Pathogenic potential within the *Brachyspira* genus was historically related to haemolysis. Pathogenic *Brachyspira* were associated with strong haemolysis, while non-pathogenic strains lacked that feature. There is some evidence that more strongly β-haemolytic strains are generally associated with greater colonic inflammation than weakly β-haemolytic ones (Burrough et al.,

2012b). However, the weakly-β-haemolytic *B. pilosicoli* is associated with colitis and diarrhea in pigs (Trott et al., 1996). To date three different genes enconding hemolysins have been associated with *Brachyspira* spp. *TlyA*. *tlyB* and *tlyC* encode gene products with the molecular masses of 26.9, 93.3 and 30.8 kDa respectively. Reports have shown that *tlyA* is required for strong haemolysis. When mutants lacking the *tlyA* gene were inoculated in mice and pigs, mild lesions were observed when compared to the wild type. Interestingly, all three genes are found in *B. hyodysenteriae* (Hsu et al., 2001; Hyatt et al., 1994; ter Huurne et al., 1994). The acyl carrier protein containing beta-haemolysin (*hylA*) has also been identified from *B. hyodysenteriae,* and haemolysis-negative *E. coli* strains containing *hlyA* became β-haemolytic on blood agar (Hsu et al., 2001). Genes encoding putative haemolysin III and a putative haemolysin CBS domain containing protein have been annotated in the whole genome sequenced of *B. hyodysenteriae* strain WA1, but no further characterization has been done (Bellgard et al., 2009). In summary, none of these genes has yet been associated with a strong immune response and the ability of causing lesions *in vivo*. At this point, this may be one of the major gaps in the SD pathogenesis, as there are yet to be described strong immunogenic virulence factors capable of inducing colitis.

Although spirochaetes reach the outer cell layer of the mucosa, there is no evidence of invasion deeper than the *lamina propria* (Glock et al., 1974; Teige and Nordstoga, 1979). This is counterintuitive, given the presence of vivid blood in the feces of affected pigs. Fibrinoid thrombi have been observed occluding superficial vessels of the *lamina propria* in damaged sites, leading to stasis and hypoxia in the surrounding areas, eventually leading to necrosis (Teige and Nordstoga, 1979). Different toxins have been suggested to contribute to such lesions (hemolysins and lipopolysaccharides), but evidence of their role in disease pathogenesis has not

yet been established (Bellgard et al., 2009; Hsu et al., 2001; Nibbelink and Wannemuehler, 1991; Wannemuehler et al., 1988).

As the SD-associated diarrhea progresses, animals become dehydrated, weak, uncoordinated, depressed and emaciated. Lethal SD cases are associated with severe dehydration, acidosis and hyperkalemia (Harris and Glock, 1971; Harris et al., 1972). In their investigation of the mechanism by which *B. hyodysenteriae* causes diarrhea, authors have reported that SD diarrhea is caused entirely by colonic absorptive failure, preceded by a sharp decrease in the lumen-to-blood fluxes of Na^+ and Cl^-. Results of these studies have also disproved the hypothesis that the abnormalities in ion transport occurred in response to increased levels of cAMP and/or cGMP (Argenzio, 1980; Schmall et al., 1983).

Diet is another factor that may contribute to development and severity of SD. The use of soybean as a dietary protein source has been linked with greater diarrhea severity due to *B. hyodysenteriae* (Jacobson et al., 2004). However, other authors have shown that dietary manipulations such as diets containing cooked rice or other highly fermentable carbohydrates resulted in acidic colon contents alter the colonic microbiota, increasing growth of microorganisms that inhibit *B. hyodysenteriae* and impair spirochaete motility, thus decreasing diarrhea severity (Durmic et al., 1998; Hampson et al., 2000; Leser et al., 2000).

The importance of the indigenous microbiota and its relationship with the host has been extensively demonstrated. Examples of this association include, but are not limited to, the prevention of mucosal colonization by pathogens, immunotolerance, maturation of enterocytes, digestion of nutrients and stimulation of peristalsis (Collado et al., 2007; Crost et al., 2010;

Durso et al., 2010; Round and Mazmanian, 2010; Round et al., 2010; Sekirov and Finlay, 2009). It is unknown what changes the colonic microbiota undergoes preceding the appearance of clinical signs due to colonization by pathogenic *Brachyspira*. Inoculation of gnotobiotic pigs with pure cultures of *B. hyodysenteriae* failed to reproduce the disease, while inoculation with colonic scrapings from clinical cases resulted in SD (Harris et al., 1978; Meyer et al., 1974a, b). These same authors observed that pigs would not develop SD when simultaneously infected with *B. hyodysenteriae* and *E. coli*, *V. coli*, or *C. perfringens*. However, when pigs were first colonized by *E. coli* and then challenged with *B. hyodysenteriae*, SD was observed. Another group reported colitis when gnotobiotic pigs were co-inoculated with *B. hyodysenteriae*, *Fusobacterium necrophorum, Bacteroides vulgatus* and *Listeria denitrificans* (Whipp et al., 1978). These results suggest that colonization of the gut and development of lesions by *B. hyodysenteriae* might rely on the presence of an indigenous microbiota.

1.8 General hypothesis

The re-emergence of mucohaemorrhagic diarrhea in western Canadian piggeries is, as identified above, a concern to producers and veterinarians. However, many any aspects of the disease are unclear at this time and require further scientific investigation in order to develop better prevention and control tools of the disease. Thus, the work described in this thesis was structured by the hypothesis that *"Brachyspira hampsonii*" is pathogenic to pigs and to porcine colon explants, leading to clinical disease *in vivo* and pathological alterations *in vitro*.

OBJECTIVES

1. To determine the pathogenicity of "*Brachyspira hampsonii*" clade I and II (strain 30599 and 30446) when inoculated in naïve pigs.

2. To determine optimal *ante-mortem* sampling tools for detection of *Brachyspira* in clinical cases.

3. To determine if any particular fecal microbiome profile is associated with higher susceptibility to development of mucohaemorrhagic diarrhea caused by *"B. hampsonii"*.

4. To determine the feasibility and optimal conditions for *in vitro* culture of swine colonic mucosa for use as a model to study infection by enteric pathogens.

5. To characterize the host tissue response to pathogenic "*Brachyspira hampsonii*" clade II (strain 30446), employing an *in vitro* organ culture model.

2 Reproduction of mucohaemorrhagic diarrhea and colitis indistinguishable from swine dysentery following experimental inoculation with "*Brachyspira hampsonii*" strain 30446

Citation

Rubin JE, Costa MO, Hill JE, Kittrell HE, Fernando C, Huang Y, O'Connor B, Harding JCS (2013) Reproduction of mucohaemorrhagic diarrhea and colitis indistinguishable from swine dysentery following experimental inoculation with "*Brachyspira hampsonii*" strain 30446. PLoS ONE 8(2): e57146. doi:10.1371/journal.pone.0057146

Author Contributions

Conceived and designed the experiments: JER JEH BO JCH. Performed the experiments: JER MOC JEH HEK CF YH BO JCH. Analyzed the data: JER MOC CF. Contributed reagents/materials/analysis tools: JEH JCH. Wrote the paper: JER MOC JEH CF BO JCH.

2.1 Abstract

Mucohaemorrhagic diarrhea caused by *Brachyspira hyodysenteriae,* swine dysentery, is a severe production limiting disease of swine. Recently, pigs in western Canada with clinical signs indistinguishable from swine dysentery were observed. Despite the presence of spirochetes on fecal smears, recognized *Brachyspira* spp. including *B. hyodysenteriae* could not be identified. A phylogenetically distinct *Brachyspira*, called "*B. hampsonii"* strain 30446, however was isolated. The purpose of this study was to experimentally reproduce mucohaemorrhagic colitis and characterize strain 30446 shedding following inoculation. Eighteen 13-week-old pigs were randomly assigned to inoculation (n=12) or control (n=6) groups in each of two trials. In trial 1, pigs were inoculated with a tissue homogenate collected from clinically affected field cases. In trial 2, pigs were inoculated with a pure broth culture of strain 30446. In both trials, mucohaemorrhagic diarrhea was significantly more common in inoculated pigs than controls, all of which remained healthy. In animals with mucohaemorrhagic diarrhea, significantly more spirochetes were observed on Gram stained fecal smears, and higher numbers of strain 30446 genome equivalents were detected by quantitative PCR (qPCR). Strain 30446 was cultured from colon and/or feces of all affected but no control animals at necropsy. *"Brachyspira hampsonii"* strain 30446 causes mucohaemorrhagic diarrhea in pigs following a 4-9 day incubation period. Fecal shedding was detectable by day 4 post inoculation, and rarely preceded the onset of mucoid or haemorrhagic diarrhea by more than 2 days. Culture and 30446-specific qPCR are reliable methods of detection of this organism in feces and tissues of diarrheic pigs. The emergence of a novel *Brachyspira* spp., such as "*B. hampsonii"*, creates diagnostic challenges

including higher risk of false negative diagnostic tests. We therefore recommend diagnostic laboratories routinely use *Brachyspira* culture, *nox*-based and species-specific PCR, and DNA sequencing to diagnose *Brachyspira*-associated colitis in pigs.

Key words: "*Brachyspira hampsonii"*; sask30446; 30446; swine dysentery; mucohaemorrhagic; bloody diarrhea; typhlocolitis

Introduction

Swine dysentery is a mucohaemorrhagic colitis causing severe production losses in pigs, resulting from infection with the intestinal spirochaete *Brachyspira hyodysenteriae*. The first clinical description of swine dysentery was published in 1921, although it was not until 1971 that *B. hyodysenteriae* (then *Treponema hyodysenteriae*) was recognized as the cause (Whiting et al., 1921a). Spirochetal colitis is a less severe illness caused by *B. pilosicoli*, which is characterized by diarrhea (non-haemorrhagic with a wet cement consistency) and poor feed conversion in chronic cases (Duhamel, 2001). Other organisms within the genus *Brachyspira* are varyingly associated with disease, including *B. murdochii, B. intermedia, B. innocens* and the provisionally named '*B. suanatina'*, although considerable strain level differences in pathogenicity (particularly among *B. intermedia*) are apparent (Jensen et al., 2010; Komarek et al., 2009; Trott et al., 1996). In October 2009, grow-finish pigs with clinical signs indistinguishable from swine dysentery were observed in a commercial barn in Saskatchewan, Canada (Harding et al., 2010a). Tissues, carcasses and rectal swabs collected from a number of affected pigs over several months were submitted to Prairie Diagnostic Services Inc. (PDS) at the University of Saskatchewan in Saskatoon, Canada. Fibrinous mucohaemorrhagic colitis and typhlitis with superficial necrosis was observed grossly. Histologically, sub-acute to chronic muco-purulent to fibrino-suppurative colitis with superficial necrosis was observed. No recognized pathogens could be identified. All samples were negative for *Lawsonia intracellularis* and *Salmonella* spp., and despite large numbers of spirochetes seen on Gram strained fecal smears, *B. hyodysenteriae* and *B. pilosicoli* were not detected. The apparent spirochetosis prompted further testing of samples by PCR using genus-specific primers targeting the *Brachyspira* NADH oxidase (*nox*) gene (Rohde et al., 2002).

The sequence of this 939 bp PCR amplicon was identical to clade 2 of the recently described, provisionally named *"Brachyspira hampsonii"* (Chander et al., 2012). The particular strain identified in western Canada and used in these trials is named 30446. Although strain 30446 is also phenotypically indistinguishable from *"B. hampsonii*" clade 2 (Chander et al., 2012), there is distinct variability within *"B. hampsonii"* (clades 1 and 2), and the pathogenicity of strain 30446 may not be reflective of all *"B. hampsonii"* isolates. As the species has not been formally recognized this study will refer precisely to *"B. hampsonii"* strain 30446.

The purpose of this study was to investigate the pathogenicity of *"Brachyspira hampsonii"* strain 30446 in experimentally infected pigs. The results of two infection trials in grower pigs, involving inoculation with either tissue homogenate (trial 1) or pure culture (trial 2) are presented.

2.2 Material and methods

2.2.1 Ethics statement

Both trials were designed and conducted in accordance with the Canadian Council for Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply (Protocol #20110038).

2.2.2 Trial 1. Tissue Homogenate Inoculation

2.2.2.1 Source of strain 30446

"Brachyspira hampsonii" strain 30446 infected material was obtained from clinically affected 13-week-old pigs from a porcine reproductive and respiratory syndrome (PRRS) negative farm. Following necropsy, the colonic and caecal mucosa were removed from the underlying sub-mucosa and muscularis by scraping with the edge of a glass microscope slide, and then frozen at -80°C within four hours of collection. To confirm the absence of pathogens other than strain 30446, sections of small and large intestine were processed routinely for histopathology, bacterial culture, and PCR.

2.2.2.2 Brachyspira culture

Brachyspira was cultured by streaking out approximately 10µg of feces or intestinal contents onto BJ and CVS agar plates (Jenkinson and Wingar, 1981; Kunkle and Kinyon, 1988). Plates were incubated anaerobically using a commercial system (Anaerogen, Oxoid Limited, Basingstoke, United Kingdom) at 42°C for 48 hours. Bacterial colonies were not formed, instead, positive cultures were indicated by zones of strong β-haemolysis from which motile spirochetes could be seen microscopically.

2.2.2.3 DNA Extraction and PCR

DNA was extracted from samples using either the QIAmp DNA stool mini kit (feces or colon contents) or DNEasy blood and tissue kit (cultured bacteria or terminal colon tissue) (Qiagen Inc., Toronto, Ontario) and 2 µl of extract used as template in PCRs. DNA was extracted in

triplicate in terminal colon tissues and colonic contents. To differentiate strain 30446 from other *Brachyspira* spp., partial 16S rRNA, cpn60, nox, adh, alp, est, gdh, glpK, pgm and thi were amplified and sequenced using previously published primers (Table 2.1).

Table 2.1. Primer sequences used to detect and identify *Brachyspira* spp.

Target Gene	Application	Primer Name	Primer Sequence (5'-3')	Reference
Nox	*Brachyspira* genus specific	NOX F	TGG CAT ACT ATC TCA TCA	(Rohde et al., 2002)
		NOX R	GAT GGA AGC TAT ATG TAT CTT A	
Adh	MLST scheme	ADH-F206	GAA GTT TAG TAA AAG ACT TTA AAC C	(Rasback et al., 2007)
		ADH-R757	CTG CTT CAG CAA AAG TTT CAA C	
Alp	MLST scheme	ALP-F354	TCC AGA TGA GGC TAT ACT TC	(Rasback et al., 2007)
		ALP-R1262	TAT GCT CTT TTT GCT AAT ATT G	
Est	MLST scheme	EST-F229	GAT GCT TCA GGC GGA GTT ATG	(Rasback et al., 2007)
		EST-R847	CCA CAC TCA TAG CAT AAA TAC TG	
Gdh	MLST scheme	GDH-F514	GGA GTT GGT GCT AGA GAG AT	(Rasback et al., 2007)
		GDH-R1157	ATC TCT AAA GCA GAA GTA GCA	
Glpk	MLST scheme	GLPK-F123	AGG CTG GGT AGA ACA TAA TGC	(Rasback et al., 2007)
		GLPK-R1158	TCT TTA CTT TGA TAA GCA ATA GC	
Pgm	MLST scheme	PGM-F172	GTT GGT ACT AAC AGA ATG AAT A	(Rasback et al., 2007)
		PGM-R1220	CCG TCT TTA TCG CGT ACA TT	
Thi	MLST scheme	THI-F163	TGT GTT ATA CAA TCA GCA CTT C	(Rasback et al., 2007)
		THI-R1079	GTA GTA AGT ATT CTA GCT CCA G	
Nox	*Brachyspira* sp. 30446 SYBR assay	JH224	TCG CTA AAT TAT TCC AAC AAG GA	This study
		JH225	AAA CGC ATT TCT ATT CCA GCA	
*cpn*60	*Brachyspira hyodysenteriae* SYBR assay	JH073	AGT GAA ATA GTT GCT CAT ATC AAA T	This study
		JH074	GCA TCA CTG ATT AAA GAA CCA AT	
*cpn*60	*Brachyspira pilosicoli* SYBR assay	JH077	ACA ATG ATA AAG AGA TAG GTG CTT	This study

To specifically detect "*B. hampsonii*" strain 30446, *B. hyodysenteriae* and *B. pilosicoli*, SYBR green qPCR assays were developed targeting either nox (strain 30446), or cpn60 (*B. hyodysenteriae* and *B. pilosicoli*) (Table 2.1). Product sizes were 215 bp for strain 30446, 120 bp for *B. hyodysenteriae* and 111 bp for *B. pilosicoli*. Quantitative PCRs were conducted on a Bio-Rad MyiQ thermocycler with iQ SYBR green supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario) according to the manufacturer's instructions. Quantification was accomplished by use of a serially diluted standard curve of plasmids containing target sequences. All reactions were run in duplicate and each run included both extraction negatives and no template controls. For samples that resulted in a Cq value higher than the lowest standard, but with dissociation curves consistent with the expected product, a result of detected but not quantifiable (DNQ) was reported. The detection limits of the assays were defined by the linear portion of the standard curve for each assay, which were 10^1-10^7 copies per reaction (10^3-10^9 copies per gram of feces or tissue) for *B. hyodysenteriae* and *B. pilosicoli*, and 10^2-10^7 copies per reaction (10^4-10^9 copies per gram of feces or tissue) for "*B. hampsonii*".

2.2.2.4 Pigs

Eighteen five-week old, Landrace male weanling piglets were purchased from a PRRS negative high health commercial farm in Saskatchewan, Canada, with no history of swine dysentery or previous laboratory diagnosis of *Brachyspira* spp. The pigs were conveniently selected, of average body weight compared to their cohorts and all appeared healthy. Farm selection was based on the screening of four and seven week old pigs from three farms for *B. hyodysenteriae, B. pilosicoli* and strain 30446 prior to trial 1. Animals were randomly assigned to control (CTRL,

n=6) and inoculated (INOC, n=12) groups on arrival, and held for a 10-day acclimation period prior to inoculation. They were fed a commercially prepared, non-medicated, pelleted starter diet ad libitum, and housed in separate rooms in 4' x 6' pens each containing 3 pigs. During the acclimation period all pigs were tested for strain 30446, *B. hyodysenteriae* and *B. pilosicoli* in feces by qPCR ten, seven and five days and immediately prior to first inoculation. Fecal DNA extracts for pre-trial screening were tested in duplicate from DNA extraction through PCR (independent technical replicates were done from DNA extraction through PCR).

2.2.2.5 Preparation of inoculum

The inoculum was prepared by mixing approximately one part mucosal scraping and three parts 0.1 M pH 7.0 phosphate buffered saline (PBS) in a sterile blender. Strain 30446 was identified in the inocula and differentiated from other *Brachyspira* species by a novel *nox* sequence (Figure 2.1) and using a previously published MLST protocol for *Brachyspira* spp. (Råsbäck et al., 2007b). Of the seven published MLST primer pairs, amplicons were not generated from three. Unique sequences were generated from *pgm* (Genbank accession JX469445, 93% sequence identity to *B. murdochii*), *thi* (JX469446, 92% sequence identity to *B. hyodysenteriae*), *glpk* (JX469444, 95% sequence identity to *B. murdochii*) and *est* (JX469443, 93% sequence identity to *B. murdochii*). The concentration of "*B. hampsonii"* strain 30446 in the inoculum was determined by qPCR and three daily inoculum doses of 3.42×10^8, 1.80×10^8 and 6.37×10^7 genome equivalents were given. These doses were intermediate to recent trials with *B. murdochii* where 10^6 colony forming units were used and a *"B. suanatina"* sp. nov. trial where 30 ml of a 10^8 to10^9 cells/mL inoculum was used (Jensen et al., 2010; Råsbäck et al., 2007a).

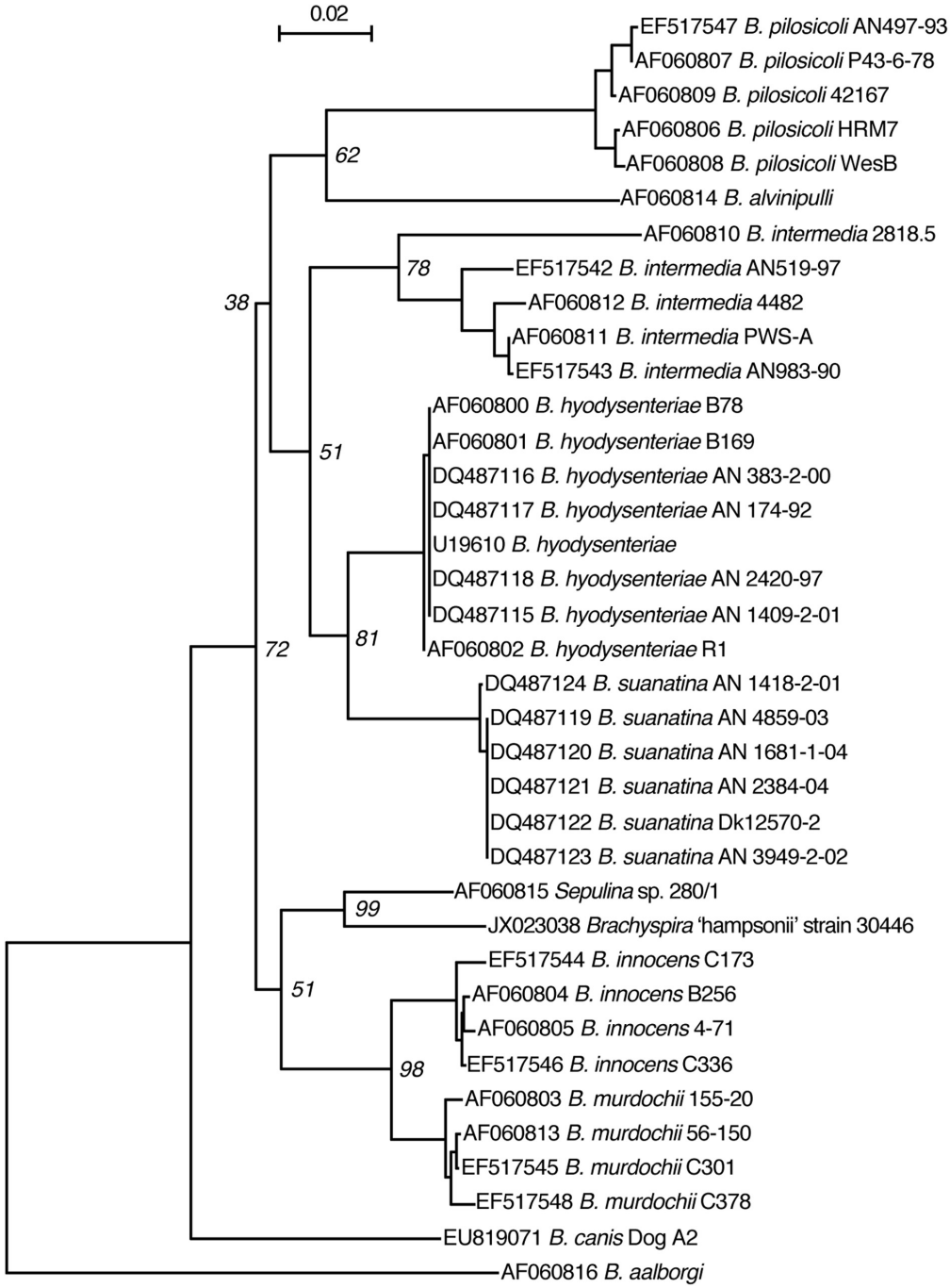


Figure 2.1. Phylogenetic tree of *Brachyspira* spp.

Phylogenetic tree based on alignment of 810 bp of the *nox* gene of *Brachyspira* spp., including *"B. hampsonii"* strain 30446. The alignment was created using CLUSTALw, followed by

distance calculation (F84 matrix) and neighbour joining using PHYLIP. The tree is a consensus of 100 boostrap iterations, and bootstrap values are indicated at the major nodes. GenBank accession numbers for *nox* sequences are indicated in the tree. Scale bar indicates 0.02 substitutions per site.

2.2.2.6 Inoculation of Pigs

Pigs were inoculated on three consecutive days (D0, D1 and D2) as previously described (Jacobson et al., 2004; Råsbäck et al., 2007a). To decrease gastric transit time, feed was removed 16 hours prior to, and returned one hour after inoculation. Prior to inoculation, pigs were sedated with azaperone 8 mg/kg IM (Stresnil, Vetoquinol Canada Inc., Lavaltrie, Quebec). Pigs in the INOC group were intra-gastrically inoculated using an 18 French feeding tube, followed by 50 mL of sterile PBS (0.1M, pH 7.0). CTRL pigs were mock inoculated with 50 mL of PBS.

2.2.2.7 Observation of Pigs and Daily Sample Collection

The pigs were observed and scored twice daily for responsiveness, skin colour, appetite and body condition, respiratory effort and fecal consistency. Fecal consistency was scored daily as: 0 = formed, normal; 1 = soft, wet cement consistency; 2 = runny or watery; 3 = mucoid diarrhea; or 4 = bloody diarrhea. Gram stained fecal smears were made from rectal swabs collected daily from each pig. An investigator blinded to the slide ID, evaluated and scored all slides as: 0 = negative; 1 = less than 1 spirochete/high power field (hpf); 2 = between 2 and 10 spirochetes/hpf; 3 = between 11 and 49 spirochetes/hpf; 4 = greater than 50 spirochetes/hpf. Fecal samples collected from each pig on days 3, 7, 10 and 14 post inoculation were also tested by qPCR for *"B. hampsonii"* strain 30446. For statistical analysis, the qPCR results were categorized: 0 = not detected; 1 = DNQ; and 2 = quantifiable.

2.2.2.8 Necropsy

After the intensity of mucohaemorrhagic diarrhea peaked (INOC), or at the end of the study (CTRL) on day 16, pigs were euthanized by cranial captive bolt and exsanguinated. A complete necropsy was performed with special attention to the stomach, duodenum, jejunum, ileum, spiral colon, caecum and rectum. Samples for histological examination, *Salmonella* culture on brilliant green agar following enrichment in selenite broth (pooled colon and caecum), *Lawsonia intracellularis* PCR (ileum)(Jones et al., 1993), porcine circovirus 2 (PCV2) immunohistochemistry (ileum, mesenteric lymph node)(Harding et al., 2008) and porcine reproductive and respiratory syndrome virus PCR (serum; Tetracore Inc., Rockville, MD) were submitted to PDS. Colonic tissue and contents were tested by qPCR for *B. hyodysenteriae*, *B. pilosicoli* and *"B. hampsonii"* strain 30446 in triplicate as done in pre-trial screening. To detect viable strain 30446, colonic tissue was cultured for *Brachyspira* and if isolated, was speciated by sequencing *nox* PCR amplicons.

2.2.2.9 Histology

The pathologist (YH) responsible for analysis of the samples was blinded to the identity of the slides. The presence or absence of superficial necrosis and inflammation were scored in Haematoxylin-Eosin stained sections of colon, caecum and rectum. Scoring for inflammation was based on the severity of neutrophil infiltration in the mucosa and fibrinous exudate on the surface. Necrosis of the mucosa was assessed by visualization of apoptotic cells and degenerated nuclei. Additionally, Warthin-Faulkner stained colon sections were examined for the presence of *Brachyspira*-like organisms associated with the lesions.

2.2.2.10 Statistics

Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL). The presence or absence of mucohaemorrhagic diarrhea in INOC vs. CTRL groups was compared using the Fisher's exact test. The presence or absence of histologic and gross lesions in INOC pigs with or without mucohaemorrhagic diarrhea, and CTRL pigs was compared using the Fisher's exact test. Spirochete slide score from colonic swabs and strain 30446 DNA concentration (0 = negative, 1 = DNQ or 2 = quantifiable) in INOC pigs with and without mucohaemorrhagic diarrhea, and in CTRL pigs were compared using the Kruskal-Wallis test followed by post-hoc Mann-Whitney test if significant. Two-tailed *P*-values ≤ 0.05 were considered significant.

2.2.3 Trial 2. Pure Broth Culture Inoculation

The methodology for trial 2 was similar to that of trial 1; only major differences will be noted below. Pigs were sourced from the same farm following pre-screening of three and six week old pigs for *B. hyodysenteriae, B. pilosicoli* and *"B. hampsonii"* strain 30446. Randomly assigned CTRL (n=6) and INOC (n=12) pigs were held for an 8 day acclimation prior to inoculation. Groups were housed in separate rooms containing 2 pigs (INOC) or 3 pigs (CTRL) per pen. During the acclimation period, feces were collected eight, five and two days, and immediately prior to first inoculation and tested for *Brachyspira* spp. by PCR and culture as described above.

"B. hampsonii" strain 30446 was cultivated *in vitro* on JBS broth from one of the pig colons used in trial 1 (isolate 6953). Approximately 2 cm^2 of solid media with haemolytic zones were

used to inoculate JBS broth (brain heart infusion with 5% (v/v) fetal calf serum, 5% (v/v) sheep's blood, and 1% (w/v) glucose). Broth cultures were incubated in glass vials with magnetic stir bars anaerobically at 39°C for 24 hours with constant stirring.

"B. hampsonii" strain 30446 was administered by intra-gastric tube for three consecutive days at doses of 2.78×10^6, 5.04×10^8 and 4.50×10^8 genome equivalents, followed by 50 ml PBS. CTRL pigs were mock inoculated with an equivalent volume of sterile JBS broth followed by 50 ml PBS. One INOC pig (#683) did not receive a complete dose of inoculum on D1, and the CTRL group inadvertently did not have feed removed prior to the second inoculation. Daily observations and fecal consistency scoring was performed as described above. The Gram stained smears made from rectal swabs were scored: 0 = less than 1 spirochete/hpf; 1 = between 2 and 10 spirochetes/hpf; 2 = between 11 and 49 spirochetes/hpf; 3 = greater than 50 spirochetes/hpf. Daily rectal swabs were cultured for *Brachyspira* and results were scored: 0 = negative; 1 = less than 10 colonies/1° streak; 2 = less than 10 colonies/2° streak; 3 = less than 10 colonies/3° streak; or 4 = less than 10 colonies/4° streak. Fecal samples collected daily from each pig were tested by qPCR for *"B. hampsonii"* strain 30446. For statistical analysis, the qPCR results were categorized: 0 = not detected; 1 = DNQ; and 2= quantifiable.

Necropsy examinations were performed after the intensity of mucohaemorrhagic diarrhea peaked (INOC), on day 14 for non-diarrheic INOC, or day 15 for CTRL. Histopathologic and microbiologic assessments of tissues were performed as described above. *Brachyspira* cultured from feces collected on the day of euthanasia and from terminal colon tissues was verified by *nox* PCR and speciated by sequencing.

Statistical analysis was identical to that described for trial 1 above. In addition, daily fecal culture results in INOC pigs with and without mucohaemorrhagic diarrhea, and in CTRL pigs were compared using the Kruskal-Wallis test followed by post-hoc Mann-Whitney test if significant.

2.3 Results

2.3.1 Source farm screening

All pigs tested at the source farm during the pre-trial screening were negative for *B. hyodysenteriae* and *B. pilosicoli* by qPCR. DNQ levels of *"B. hampsonii"* strain 30446 were found in 1/20 pigs prior to trial 1 and 3/20 pigs prior to trial 2. As DNQ levels of strain 30446 were detected at all three potential source farms tested, the farm with the lowest prevalence was used to supply the pigs for both trials. The pre-trial screening was conducted on different pigs from those used in each experiment.

2.3.2 Trial 1. Tissue Homogenate Inoculation

During the acclimation period, 3/12 INOC pigs had DNQ levels of strain 30446 DNA present in feces at single time points (Table 2.2). The study continued for 16 days following the first inoculation. All CTRL animals remained healthy throughout the study, and spirochetes were never seen in the feces nor detected by qPCR. In some INOC pigs, strain 30446 was detected in feces at very low levels (DNQ) on day 3 post inoculation (PI) (#53, #54, #55, #65), day 7 PI (#64) and at quantifiable levels on day 7 PI (#64; 2.67×10^5 genome equivalents/g) and day 10

PI (#68; 4.45 × 10^7 genome equivalents). Of the 12 INOC pigs, nine developed mucohaemorrhagic diarrhea between days 4 and 10 PI (Figure 2.2). Of the remaining three INOC pigs, two (#61 and #63) developed soft feces on days nine and ten, which resolved on days 12 and 14 PI respectively. Pig #54 developed watery diarrhea on day four PI. Neither blood nor mucous were observed from any of these three animals. Mucohaemorrhagic diarrhea was significantly more common in INOC (9 of 12) than CTRL (0 of 6) ($P < 0.001$).

Table 2.2. Detection of *"B. hampsonii"* strain 30446 in pre-inoculation screening of fecal samples during the acclimation period[1]

Trial 1				**Trial 2**				
Pig ID	**Day -10**	**Day -7**	**Day -5**	**Pig ID**	**Day -8**	**Day -5**	**Day -2**	**Day 0**
Inoculated				**Inoculated**				
51	-	-	-	683	-	-	-	-
53	-	-	-	684	-	-	-	6.80×10^4
54	-	-	-	686	-	-	-	-
55	-	-	-	688	DNQ	-	-	-
57	-	-	-	689	DNQ	-	-	-
59	DNQ	-	-	690	-	-	DNQ	DNQ
61	-	-	-	693	-	-	-	-
63	-	-	-	694	-	-	DNQ	-
64	-	-	DNQ	695	-	-	-	DNQ
65	-	-	-	696	DNQ	-	DNQ	DNQ
67	-	-	DNQ	697	DNQ	-	-	-
68	-	-	-	700	DNQ	-	-	DNQ
Control				**Control**				
52	-	-	-	685	-	-	-	DNQ
56	-	-	-	687	DNQ	-	-	-
58	-	-	-	691	DNQ	-	-	DNQ
60	-	-	-	692	-	-	-	-
62	-	-	-	698	-	-	-	-
66	-	-	-	699	-	-	-	DNQ

[1]Quantitative PCR results for all pigs at ten, seven and five days prior to inoculation in trial 1, and eight, five and two days prior to inoculation, and at inoculation in trial 2. Hyphens indicate that strain 30446 was not detected, DNQ indicates detectable but not quantifiable concentrations, and a number indicates the concentration of strain 30446 in genome equivalents/g of feces.

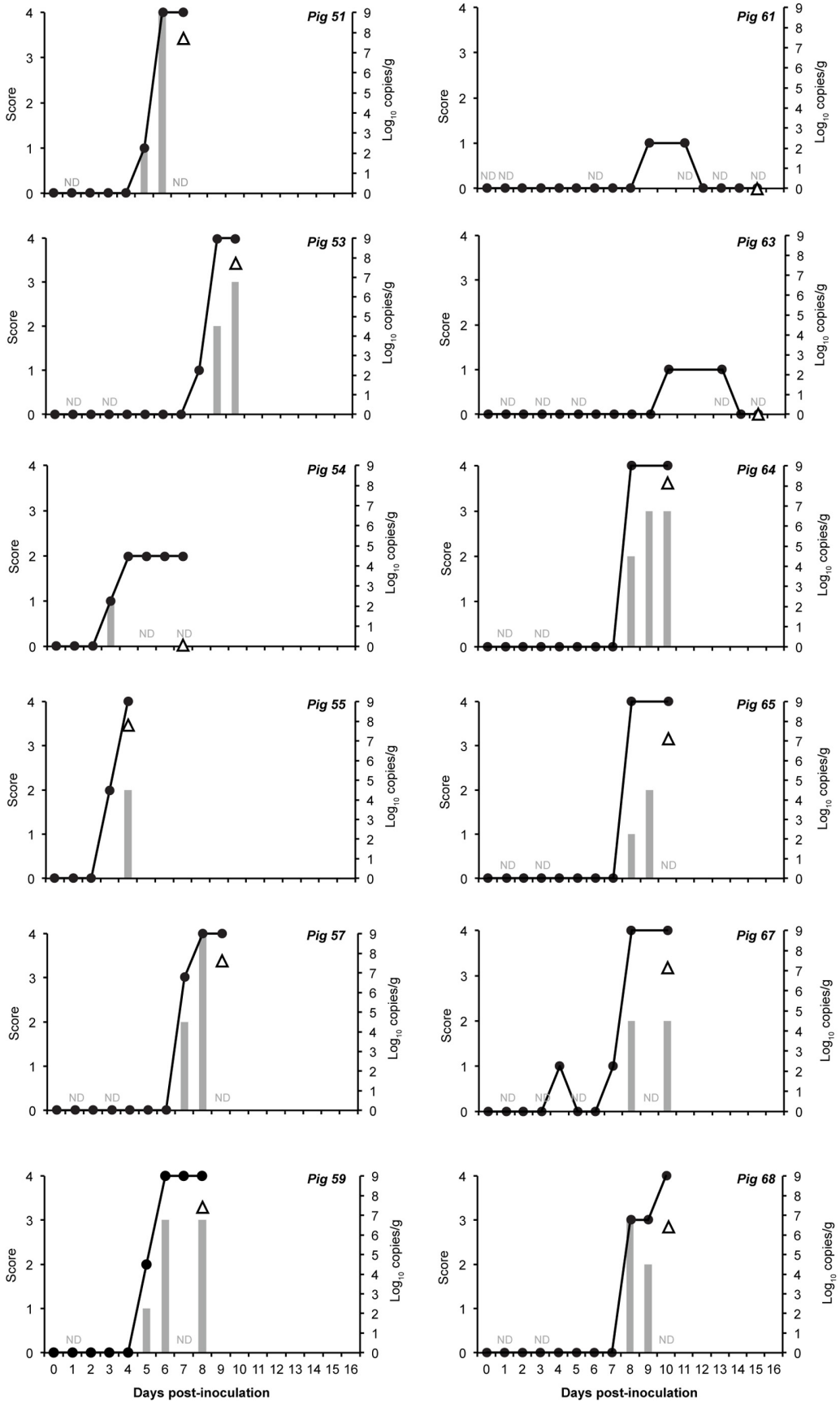

Pig 51
Pig 61
Pig 53
Pig 63
Pig 54
Pig 64
Pig 55
Pig 65
Pig 57
Pig 67
Pig 59
Pig 68
Score
Log10 copies/g
ND
Days post-inoculation

Figure 2.2. Fecal consistency, shedding and tissue concentrations in pigs following inoculation with tissue homogenate containing *"Brachyspira hampsonii"* strain 30446.

Daily fecal consistency scores (line, left ordinate; 0 = formed, normal; 1 = soft, wet cement consistency; 2 = runny or watery; 3 = mucoid diarrhea; or 4 = bloody diarrhea). Fecal smear spirochete scores (grey bars, left ordinate: 0 = negative; 1 = less than 1 spirochete/high power field (hpf); 2 = between 2 and 10 spirochetes/hfp; 3 = between 11 and 49 spirochetes/hpf;4 = greater than 50 spirochetes/hpf). Strain 30446 DNA concentration (copies/g, triangles, right ordinate) in colon tissue samples collected at necropsy. Pig IDs are indicated in the upper right corner of each panel. ND = fecal smear spirochete score not done.

In pigs that developed mucohaemorrhagic diarrhea, the number of spirochetes seen on Gram stained slides increased concurrently with elevated fecal consistency scores (Figure 2.2). In one INOC pig (#67), transient mild diarrhea was observed four days before the first spirochetes were seen, and three days prior to the onset of persistent diarrhea (Figure 2.2). Of the three INOC pigs that did not develop mucohaemorrhagic diarrhea, spirochetes were not observed from two (#61 and #63), transient low level spirochete shedding was observed on a single day in the other (#54) (Figure 2.2). Significantly higher terminal spirochete slide scores were seen in pigs with mucohaemorrhagic diarrhea than either INOC pigs without mucohaemorrhagic diarrhea (P = 0.011) or CTRL ($P < 0.001$). Similarly, in terminal colon, strain 30446 concentrations were significantly higher in pigs with mucohaemorrhagic diarrhea (1.84×10^7 - 1.32×10^8) than either INOC without mucohaemorrhagic diarrhea (negative - DNQ, $P = 0.009$) or CTRL (negative, $P <$ 0.001). Neither *B. hyodysenteriae* nor *B. pilosicoli* were detected in terminal colon tissue or contents from any pig.

Lawsonia intracellularis, B. hyodysenteriae, B. pilosicoli, Salmonella spp.*,* and PRRS virus were not detected in terminal samples from any pig. A single INOC pig (#57) was weakly positive for PCV2, and all other animals were negative. Culture of colonic tissue revealed *"B. hampsonii"* strain 30446 in nine, and *B. intermedia* (98% identical over 805 bp of the *nox* gene to *B. intermedia* ATCC 51140^T) in one (#54) INOC pigs. Two INOC pigs (#61 and #63) were culture negative. The *nox* sequence of an isolate similar to *B. intermedia* (97% identical over 824 bp of the *nox* gene to *B. intermedia* ATCC 51140^T) was also isolated by culture from one CTRL pig (#56).

Gross pathological findings were consistent with clinical signs. Lesions were significantly more common in pigs with mucohaemorrhagic diarrhea than in INOC pigs without mucohaemorrhagic diarrhea (Table 2.3). In affected pigs, enlargement of the mesenteric lymph nodes, fibrinous typhlocolitis, meso-colonic edema and/or congestion and abundant mucohaemorrhagic caecal and rectal contents were seen. Small intestinal lesions were seen in four INOC pigs including serosal congestion of the ileum (n=1) and jejunum (n=3). Hyperkeratosis of the gastric epithelium was seen in 14 pigs (INOC n=8 and CTRL n=6). Mild to moderate erosions were seen in the pars esophagea of 5 pigs, one INOC with mucohaemorrhagic diarrhea, one INOC without mucohaemorrhagic diarrhea, and three CTRL.

Table 2.3. Comparison of histological and gross lesions and spirochete numbers on Warthin-Faulkner stained colonic sections[1]

Experiment		Histologic Lesions			Gross Lesions			Spirochetes on Silver Stain	
		Colon	Caecum	Rectum	Colon Mucoid and/or haemorrhagic colitis	Edema and congestion	Caecum Muco-fibrinous typhlitis	Absent-Rare	Abundant
Tissue Homogenate Trial 1	Control	0/6†	0/6†	0/6†	0/6†	0/6†	0/6†	2/6	0/6
	Inoculated	9/12	5/12	6/12	7/12	9/12	7/12	3/12	9/12
	Diarrheic	9/9*†	5/9†	5/9†	7/9*†	9/9*†	7/9*†	0/9	9/9
	No diarrhea	0/3*	0/3	1/3	0/3*	0/3*	0/3*	3/3	0/3
Pure Culture Trial 2	Control	0/6‡	0/6‡	0/6‡	0/6‡	0/6‡	0/6‡	6/6	0/6
	Inoculated	7/12	6/12	8/12	7/12	5/12	5/12	4/12	8/12
	Diarrheic	7/8$^{\#\ddagger}$	6/8‡	8/8$^{\#\ddagger}$	7/8$^{\#\ddagger}$	5/8‡	5/8‡	0/8	8/8
	No diarrhea	0/4$^{\#}$	0/4	0/4$^{\#}$	0/4$^{\#}$	0/4	0/4	4/4	0/4

[1]Statistical comparisons were among CTRL (n=6), INOC with mucohaemorrhagic diarrhea (n=9) and INOC without mucohaemorrhagic diarrhea (n=3) in trial 1. In trial 2, comparisons were among CTRL (n=6), INOC with mucohaemorrhagic diarrhea (n=8) and INOC without mucohaemorrhagic diarrhea (n=4) in trial 2. Significant differences ($P \leq 0.05$) are denoted by different superscript symbols within column.

Histologically, necrosis of the superficial colonic mucosa, which was covered by a bacteria-rich, mucoid exudate that extended into the superficial crypts, was observed. When examined using Warthin-Faulkner silver stains, numerous long, thin spirochetes were seen in the crypts and among the mixed bacteria on the surface (Figure 2.3). There was mild but variable congestion and haemorrhage in the lamina propria, and a mild neutrophil infiltration in the lamina propria and lumen in some cases. Lesions were most consistent in the colon but were also detected in the caecum and rectum. Lesions were significantly more common in pigs with mucohaemorrhagic diarrhea than in either INOC without mucohaemorrhagic diarrhea or CTRL (Table 2.3).

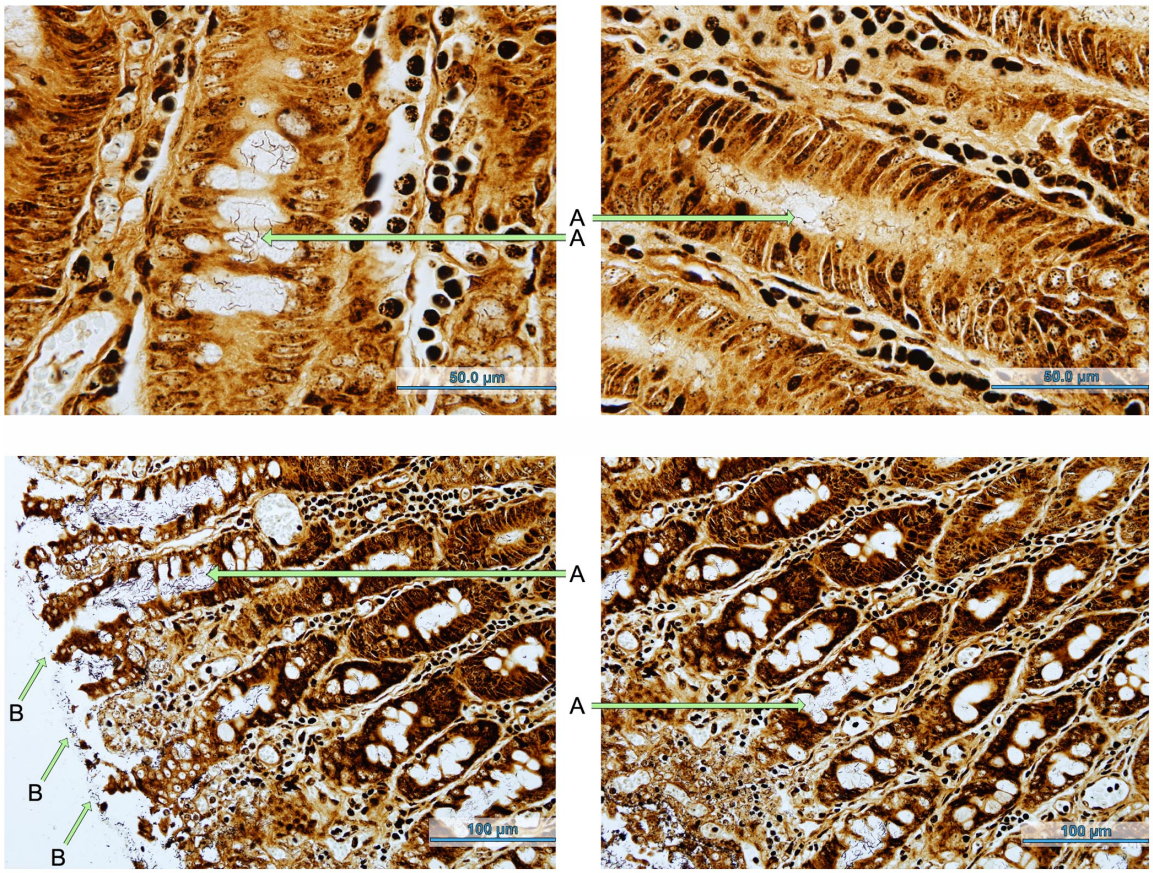


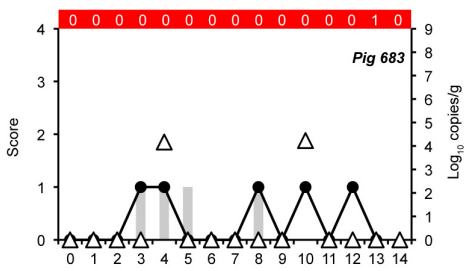
Figure 2.3. *"Brachyspira hampsonii"* strain 30446 in Warthin-Faulkner stained colonic sections.

Histologic sections of spiral colon taken from pigs with mucohaemorrhagic diarrhea examined with Warthin-Faulkner silver staining. Spirochetes can be seen in the intestinal crypts (A) and along the epithelial surface (B).

2.3.3 Trial 2. Pure Broth Culture Inoculation

"B. hampsonii" strain 30446 was detected at DNQ levels in various INOC (8 of 12) and CTRL (4 of 6) pigs during the acclimation period, but generally as single events (Table 2.2). One INOC pig (#684) had a quantifiable level of strain 30446 (6.80×10^4 copies/g) on the day of inoculation.

The study continued for 14 days following the first inoculation. All CTRL animals remained healthy throughout the study. Although low numbers of spirochetes were seen by direct microscopic examination in feces of CTRL pigs, no sample was positive by qPCR or culture during following inoculation. Of the 12 INOC pigs, eight developed mucohaemorrhagic diarrhea between 5 and 9 days PI (Figures 2.4 to 2.6). Of the remaining four INOC pigs (#683, #684, #688 and #695) soft feces were variably observed between days 1 and 14. Low levels of strain 30446 shedding (1.48×10^4 to 2.67×10^5 genome copies/g) were detected by qPCR in the feces of three of these pigs (#683, #684 and #695), and positive culture results were obtained for #683, #688 and #695. Mucohaemorrhagic diarrhea was significantly more common in INOC (8 of 12) than CTRL (0 of 6) ($P = 0.013$).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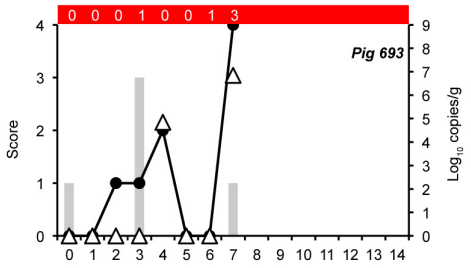
Pig 683
Score
Log10 copies/g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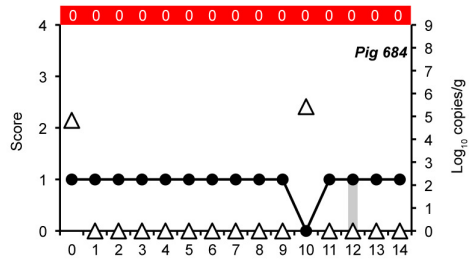
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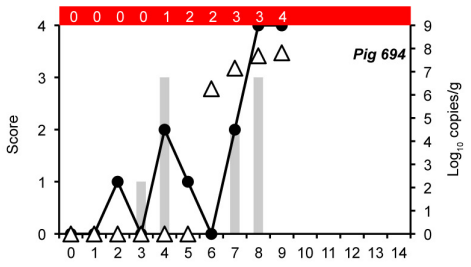
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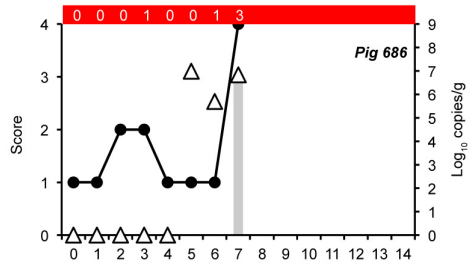
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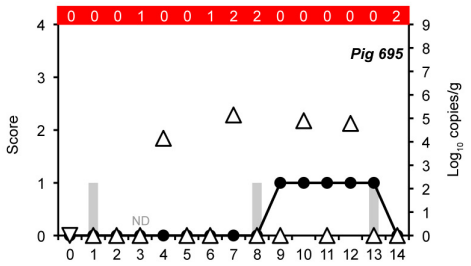
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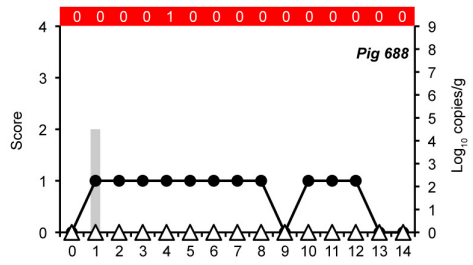
Score
Log10 copies/g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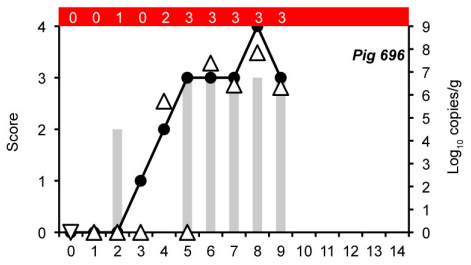
Log10 copies/g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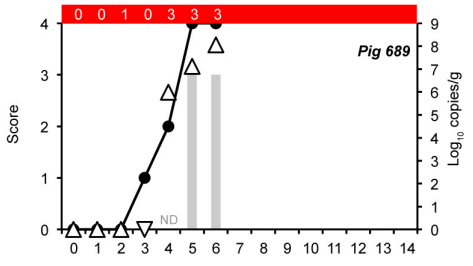
Log10 copies/g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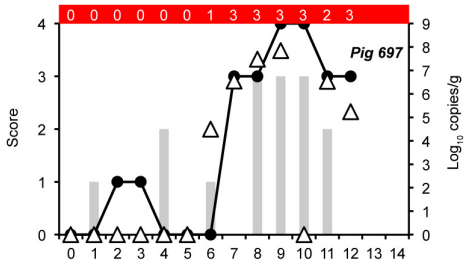
Pig 689
ND
Score
Log10 copies/g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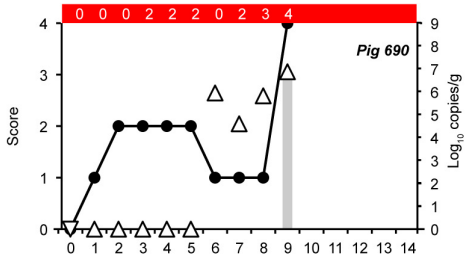
Pig 697
Score
Log10 copies/g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Pig 690
Score
Log10 copies/g

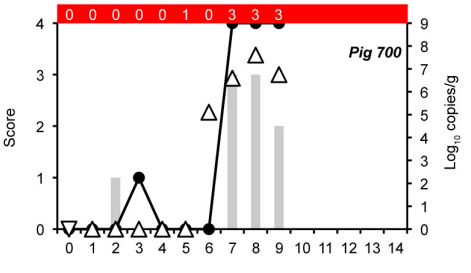

0 0 0 0 0 1 0 3 3 3
Pig 700
Score
Log10 copies/g

Figure 2.4. Fecal consistency, shedding and tissue concentrations in pigs following inoculation with pure broth cultivated "*Brachyspira hampsonii*" strain 30446.

Fecal consistency scores (line, left ordinate: 0 = formed, normal; 1 = soft, wet cement consistency; 2 = runny or watery; 3 = mucoid diarrhea; or 4 = bloody diarrhea). Fecal smear spirochete scores (grey bars, left ordinate: 0 = less than 1 spirochete/high power field (hpf); 1 = between 2 and 10 spirochetes/hpf; 2 = between 11 and 49 spirochetes/hpf; 3 = greater than 50 spirochetes/hpf). Strain 30446 DNA concentration in feces (triangles, right ordinate), upside down triangles indicate DNQ. The red bar at the top of each panel indicates the semi-quantitative fecal culture score (0 = negative; 1 = less than 10 colonies/1° streak; 2 = less than 10 colonies/2° streak; 3 = less than 10 colonies/3°streak; 3 = less than 10 colonies/4° streak). Pig IDs are indicated in the upper right corner of each panel. ND = fecal smear spirochete score not done.


A


B

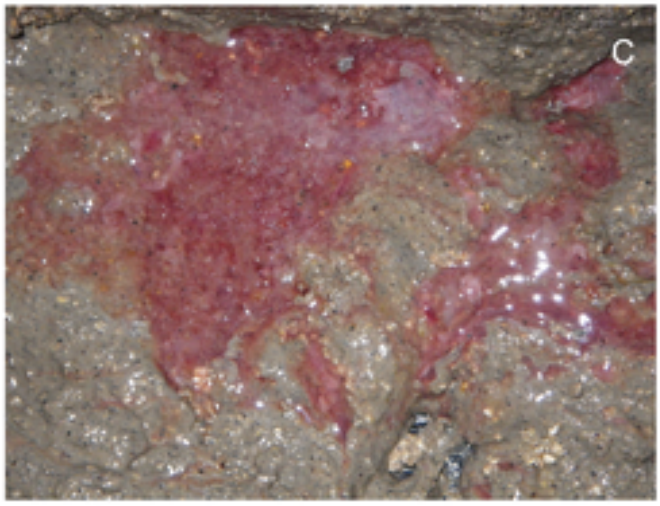

C

Figure 2.5. Mucohaemorrhagic diarrhea associated with "*Brachyspira hampsonii*" strain 30446.

Fecal consistency following inoculation with pure broth "*Brachyspira hampsonii*" strain 30446 culture ranged from that similar to wet cement (A, right side), clots of blood (A, left side) or mucus (B), or severe watery mucohaemorrhagic diarrhea (C). Images A, B, C were taken on days 5, 6 and 8 PI respectively.

Figure 2.6. Perianal fecal staining.

In some pigs, mucohaemorrhagic feces adheres to the perianal region of pigs following inoculation with pure broth "*Brachyspira hampsonii"* strain 30446. Both images are taken on day 8 PI: frank blood with a clot (A), blood with mucus (B).

While fecal consistency score, spirochete slide score, culture results and qPCR counts varied daily, when elevated, they tended to move together as a group (Figure 2.4**)**. Gram stain slide scores of 3+ were seen before or concurrently with diarrhea among pigs that developed mucohaemorrhagic diarrhea, whereas scores above 2+ were not observed in pigs without mucohaemorrhagic diarrhea. Similarly, 2+ or 3+ cultures were only observed in pigs that developed mucohaemorrhagic diarrhea (Figure 2.4). Compared to CTRL or INOC without mucohaemorrhagic diarrhea, INOC pigs with mucohaemorrhagic diarrhea had significantly higher terminal spirochete slide scores ($P = 0.009$ for INOC with diarrhea, $P = 0.026$ for INOC without diarrhea) and more frequent isolation of 30446 by culture ($P = 0.001$ for INOC with diarrhea, $P = 0.004$ for INOC without diarrhea). A significantly higher number of genome equivalents of strain 30446 DNA was detected by qPCR in the colonic tissue of pigs with mucohaemorrhagic diarrhea than CTRL ($P < 0.001$). Although the same association was seen between pigs with mucohaemorrhagic diarrhea (9.65×10^3 - 1.85×10^7 genome copies/g) and INOC without mucohaemorrhagic diarrhea (DNQ - 1.03×10^4 genome copies/g), the non-parametric method of analysis was insensitive to this difference ($P = 0.157$). Sequencing of *nox* PCR amplicons from terminal fecal and colon tissue cultures revealed isolates with 99-100% sequence similarity to strain 30446 in all pigs with mucohaemorrhagic diarrhea and one INOC pig without mucohaemorrhagic diarrhea (#684). An isolate with sequence similarity to *B. intermedia* (97% identity over 825 bp of the *nox* gene to *B. intermedia* ATCC 51140^T) was grown from one other INOC pig without mucohaemorrhagic diarrhea (#695). All CTRL pigs were culture negative. Importantly, *nox* sequence of the *Brachyspira* species isolated from the

terminal fecal samples from the INOC group matched the *nox* sequences of the isolates from terminal colon culture.

Lawsonia intracellularis, B. hyodysenteriae, B. pilosicoli, Salmonella spp. and PRRS were not detected in terminal samples from any pig. Pig #695 tested positive for PCV2 in ileum and lymph node by immunohistochemistry, while all others tested negative.

2.3.4 Necropsy

Gross pathological findings were consistent with the clinical signs. Lesions were significantly more common in pigs with mucohaemorrhagic diarrhea than in INOC pigs without mucohaemorrhagic diarrhea or CTRL (Table 2.3). In affected pigs, lesions were typified by mucoid or mucohaemorrhagic colitis and fibrino-mucoid typhlitis. Lesions ranging in severity from mild congestion to severe fibrino-necrotic colitis with profuse mucous were seen (Figure 2.7). Small intestinal lesions were found in three pigs with mucohaemorrhagic diarrhea and one INOC without mucohaemorrhagic diarrhea, and consisted of mild corrugation and thickening of the ileum grossly. Hyperkeratosis of the pars esophagea was seen in 14 pigs (INOC n=8, CTRL n=6). Additionally, one pig with mucohaemorrhagic diarrhea had a well demarcated area of superficial necrosis in the gastric fundus, and one INOC pig without mucohaemorrhagic diarrhea had small erosions of the pars esophagea.

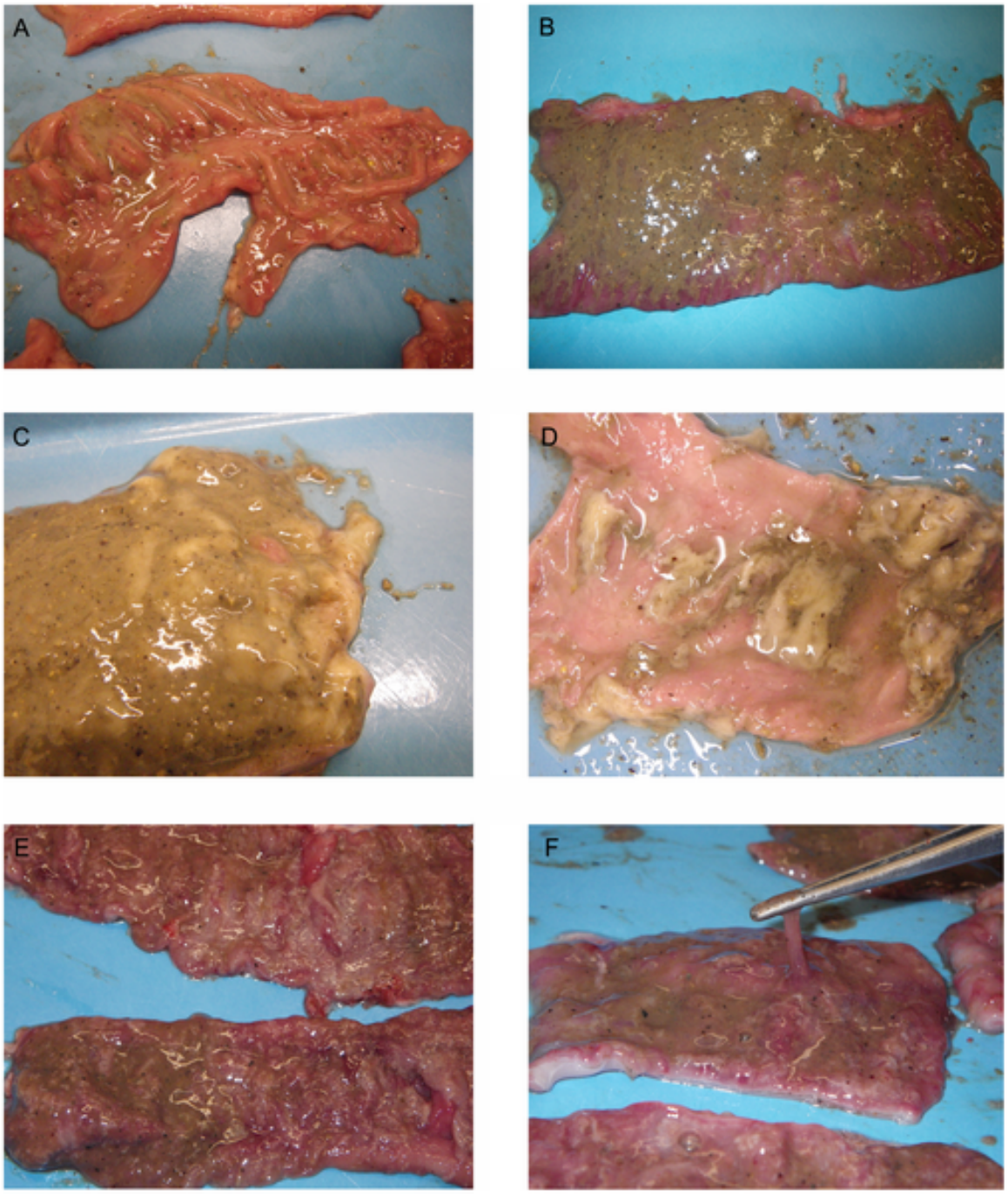


Figure 2.7. Colonic mucosal lesions associated with "*Brachyspira hampsonii*" strain 30446.

Gross colonic mucosal lesions observed following inoculation with pure broth culture containing strain 30446. Images are of different INOC pigs euthanized between D6 and D12 post-inoculation. A = mild to moderate hyperemia and/or congestion with scant mucous deposited on mucosal surface;

B = moderate to severe mucosal congestion with normal looking contents adhering to mucosa; C = moderate to severe fibrinomucoid exudate adhering to hyperemic mucosal surface prior to washing; D = patchy fibrinomucoid exudate adhering to hyperemic mucosal surface after washing; E = severe fibrinonecrotic colitis; F = thick adherent mucoid exudate on mucosal surface of colon.

The distribution of histologic lesions was significantly associated with clinical signs and was similar to that observed in trial 1 (Table 2.3). There were no abnormal findings in either CTRL or INOC without mucohaemorrhagic diarrhea. Inflammation and/or necrosis of the superficial mucosa of the colon, caecum and rectum were seen in all affected pigs.

Amplicons were generated from *nox* PCR from terminal colon tissue from all INOC pigs. Sequencing revealed 11 amplicons with 99-100% sequence identity to strain 30446 (including all affected pigs) and one amplicon with 97% sequence identity to *B. intermedia* ATCC 51140^T (from #695, an INOC pig that did not develop mucohaemorrhagic diarrhea). When tested by qPCR, strain 30446 specific amplicons were generated for all 12 INOC pigs and no CTRL pigs. When sequenced, 10 of these amplicons were 99-100% similar to strain 30446, while sequencing failed for the other two. The colon contents from one CTRL pig (#685) were positive for *B. pilosicoli* by qPCR (1.65×10^4 copies/g of feces) and its identity was confirmed by sequencing. Neither *B. hyodysenteriae* nor *B. pilosicoli* were detected in any other pigs.

2.4 Discussion

Swine dysentery, which by definition is caused by *B. hyodysenteriae* (Taylor and Alexander, 1971), has recently undergone a period of relative quiescence in North America. The emergence of a clinically indistinguishable illness associated with a distinct organism, "*B. hampsonii"* strain 30446 poses diagnostic challenges for producers, veterinarians and diagnosticians (Burrough et al., 2012b; Gebhart et al., 2012; Harding et al., 2010a; Harding et al., 2010b). The lack of pathognomonic clinical or pathological findings associated with "*B. hampsonii"* strain 30446

necessitates a reliance on laboratory tests to make a specific etiological diagnosis. Unfortunately there is no standardized method of identification. Although there have been no attempts to standardize testing amongst laboratories to date, the use of direct examination, culture followed by genus specific PCR and sequencing, and species-specific PCR are recommended. A number of *Brachyspira* species specific PCR assays have been published (Burrough et al., 2012b; Råsbäck et al., 2005; Song and Hampson, 2009) but due to the apparent widespread use of techniques developed in house or modifications to published protocols, the relative sensitivity and specificity of these assays are unknown. Thus, when using PCR, negative results should be interpreted with caution. In light of these challenges, evaluation of fecal smears may be invaluable diagnostically. While fecal smears cannot differentiate between *Brachyspira* species, the presence or absence of spirochetes is useful for interpreting molecular test results.

This research confirms that *"Brachyspira hampsonii"* strain 30446 induces a mucohaemorrhagic diarrhea and colitis in pigs that is indistinguishable from swine dysentery. Trial 1 preceded our ability to grow "*B. hampsonii"* strain 30446 in broth culture, but the development of JBS broth in October 2011 made it possible to use a pure culture inoculum in trial 2. This pure broth culture was prepared from the tissue inoculum used in trial 1. The results of trial 2 demonstrate that strain 30446 causes mucohaemorrhagic diarrhea indistinguishable from the field cases where it was first observed, establishing this organism as a pathogen of swine.

The design of these trials was based on a successful, previously published trial utilizing *B. murdochii* (Jensen et al., 2010). Because the minimum infectious dose of strain 30446 is unknown, average doses of 1.95×10^8 genome equivalents in trial 1 and 3.19×10^8 genome

equivalents in trial 2 were used, intermediate to those used in previous studies. Disease developed between four and ten days PI in trial 1 and four to nine days PI in trial 2 indicating that a dose sufficient to cause disease was used. Interestingly, the incubation period observed in these trials was consistent with an infection trial conducted as part of the initial description of swine dysentery in 1921 (Whiting et al., 1921b).

Pre-screening fecal samples of experimental pigs revealed a low level of "*B. hampsonii"* strain 30446 colonization (reported as DNQ) in 3/18 pigs in trial 1, and 12/18 pigs in trial 2 based on qPCR. In addition, 1 pig in trial 2 (#684) had 6.80×10^4 genome equivalents/g of "*B. hampsonii"* strain 30446 in feces on D0 (Table 2.2). This finding suggests that strain 30446 is not an obligate pathogen but instead causes disease when present in sufficient numbers or when host defences are compromised. The use of strain 30446 negative pigs was preferable, but none were available at the time this research was undertaken. Low (DNQ) pre-challenge levels of *"B. hampsonii"* strain 30446 however, clearly did not induce sufficient mucosal immunity to protect against disease as evidenced by the 3/3 trial 1 pigs and 6/8 trial 2 pigs that developed mucohaemorrhagic diarrhea following challenge. Noteworthy is pig #684 that did not develop mucohaemorrhagic diarrhea following challenge, suggesting that shedding $\sim 10^4$ genome equivalents/g feces may have been associated with sufficient mucosal immunity to protect against disease. The results of a number of diagnostic cases completed by our team demonstrates a similar trend whereby clinical cases typically have greater than 10^5 *"B. hampsonii"* strain 30446 genome equivalents/g of tissue or feces, whereas age-matched non-clinical animals in the same airspace have fewer than 10^5 genomic copies/g (Harding et al., 2011). Pre-trial colonization of CTRL animals, all of which remained healthy, supports the conclusion that DNQ levels of

strain 30446 in feces are incidental. These findings are consistent with a previous report that concluded that concentrations of *B. hyodysenteriae* greater than 10^5 CFU/gram of feces are required for the development of lesions (Wilcock and Olander, 1979).

Three pigs in trial 1 and four pigs in trial 2 did not develop mucohaemorrhagic diarrhea in spite of being inoculated. In trial 2, one pig (#683) only received two of three inoculum doses, and one (#684) was the pig with 10^4 copies/g strain 30446 on D0. Whether failure to produce disease in these seven animals reflects normal biological variation, pre-existing immunity or the use of a marginally infectious dose is unknown.

Pathological findings in affected pigs, characterized by mucoid or mucohaemorrhagic colitis and muco-fibrinous typhlitis, were consistent with previous reports describing swine dysentery (Kinyon et al., 1977). Histopathological findings in the caecum and colon of affected pigs were consistent with, but mild in comparison to gross lesions. The mild thickening and congestion seen in the ileum of four INOC pigs (one unaffected and three with mucohaemorrhagic diarrhea) in trial 2, mimicked early or mild proliferative ileitis caused by *L. intracellularis,* however the ileum of all pigs were negative by PCR for *L. intracellularis* and in no pigs were lesions typical of ileitis seen histologically. Whether or not these mild ileal lesions are a feature of strain 30446 associated disease or an incidental finding is unknown.

A number of atypical or novel, phylogenetically distinct, strongly β-haemolytic *Brachyspira* have been reported to cause disease in pigs (Burrough et al.; Burrough et al., 2012b). Based on partial *nox* sequence, strain 30446 clusters separately from the other known species with good bootstrap support (Figure 2.1). The partial *nox* sequence for strain 30446 is identical to the

provisionally named *"B. hampsonii"*, and 94.8% similar over 810 bp to *Serpulina* sp. P280/1, a porcine clinical isolate from the United Kingdom (Atyeo et al., 1999; Chander et al., 2012; Neef et al., 1994). It is approximately 92% identical to the *B. innocens,* and *B. murdochii* strains examined, which is less similar than these two species are to each other (pairwise identities between *B. innocens* and *murdochii* are 96-97% over this same region). Furthermore, three of seven primer sets from a previously published MLST scheme for *Brachyspira* sp, failed to yield a product, while novel sequences were generated for the other four.

Two recent reports describe infection experiments using murine (Burrough et al.; Burrough et al., 2012b) and porcine (Burrough et al., 2012b) models of swine dysentery with North American *Brachyspira* strains including "*B. hampsonii"* strain 30446 isolated from pigs with signs of swine dysentery in Iowa between 2008 and 2011. These experiments were the first to demonstrate the causal relationship between strain 30446 and mucohaemorrhagic typhlocolitis in pigs. The authors found that strongly β-haemolytic strains of *Brachyspira* spp., including strain 30446, produced disease and colonic lesions typical of those associated with *B. hyodysenteriae.* In the porcine experiment (Burrough et al., 2012a; Burrough et al.), 4 of 10 pigs inoculated with strain 30446 had diarrhea on days 7 and 14 post infection, and 2 of 10 were culture or PCR positive at necropsy on day 16. By contrast, the incidence of mucohaemorrhagic diarrhea reported here in trial 1 and 2 was 75% and 67% respectively in INOC pigs. Furthermore, following inoculation with pure broth culture, strain 30446 was isolated by culture from 10/12 pigs, was detected by PCR in feces in 11/12 pigs and repeatedly for 3 or more days in 8 pigs. Testing for other relevant pathogens including PRRS virus, *Lawsonia intracellularis and Salmonella* spp., were negative. Collectively, these data provide substantive evidence of

causality and provide the first report characterizing fecal shedding using a Canadian "*B. hampsonii"* 30446 isolate. While strain 30446 used for the present research clearly falls within *"B. hampsonii"* clade 2, the relationship between this strain and other members of the species, particularly clade 1, is unclear. Hence, additional research is necessary to more fully understand the clinical relevance of this novel and diverse *Brachyspira* species in swine.

In summary, our results confirm the causal relationship between *"Brachyspira hampsonii"* strain 30446 and mucohaemorrhagic diarrhea in swine. The emergence of *"Brachyspira hampsonii"* strain 30446 therefore poses potential diagnostic challenges since the specificity of some currently used PCR assays may not detect this organism, and *Brachyspira* culture is not widely used in diagnostic laboratories in many countries. To date, cases have been diagnosed in pigs from Alberta, Saskatchewan, Iowa, Illinois, Minnesota, Missouri and North Carolina (Chander et al., 2012; Harding et al., 2010a). The prevalence of carrier animals, risk factors for infection, non-porcine reservoirs, antimicrobial susceptibility, minimal infectious dose and the efficacy of cleaning practices for eliminating *"B. hampsonii"* strain 30446 are entirely unknown. Research is ongoing in our lab to address these and other important questions.

2.5 Transition statement

The experiments described in this chapter showed that inoculation of naïve pigs with "*B. hampsonii*" strain 30446 culminates in mucohaemorrhagic diarrhea and shedding of the microorganism by the host. This novel species was isolated from pigs that had clinical signs indistinguishable from *B. hyodysenteriae* infection. However, we and other authors have identified other strains of "*B. hampsonii*" from field cases of mucohaemorrhagic diarrhea. Using a genus-specific PCR, isolates were characterized and are now classified as clades I and II (Chander et al., 2012). Results from this chapter confirmed that inoculation with a clade II isolate (30446) led to disease after a 4-9 day incubation period. Next, a study was designed to test the hypothesis that "*B. hampsonii*" clade I was also an agent of mucohaemorrhagic diarrhea in pigs. In addition, we tested the performance of different *ante-mortem* sampling methods for detection of the pathogen.

3 Confirmation that "*Brachyspira hampsonii*" clade I (Canadian strain 30599) causes mucohaemorrhagic diarrhea and colitis in experimentally infected pigs

Citation

Costa MO, Hill JE, Fernando C, Lemieux HD, Detmer SE, Rubin JE, Harding JCS (2014) Confirmation that "*Brachyspira hampsonii*" clade I (Canadian strain 30599) causes mucohaemorrhagic diarrhea and colitis in experimentally infected pigs. BMC Vet Res. 2014; 10:129.

Author Contributions

In vivo work was conducted by HDL, MOC and JCSH. Laboratory work was conducted by MOC, CF, HDL and JEH. MOC evaluated the ante mortem sample methodologies. SED performed pathological assessments. JEH and JCSH are Principal Investigators.

3.1 Abstract

"*Brachyspira hampsonii*", discovered in North America in 2010 associated with dysentery-like illness, is an economically relevant swine pathogen resulting in decreased feed efficiency and increased morbidity, mortality and medication usage. *"B. hampsonii"* clade II strain 30446 has been shown to be causally associated with mucohaemorrhagic diarrhea and colitis. Our objectives were to determine if "*Brachyspira hampsonii*" clade I strain 30599 is pathogenic to pigs, and to evaluate the relative diagnostic performance of three *ante mortem* sampling methodologies (direct PCR on feces, PCR on rectal GenoTube Livestock swabs, *Brachyspira* culture from rectal swabs). Five-week old pigs were intragastrically inoculated thrice with 10^8 genomic equivalents *"B. hampsonii"* (n=12), or served as sham controls (n=6). Feces were sampled and consistency assessed daily. Necropsies were performed 24h after peak clinical signs. One pig died due to unrelated illness. Nine of 11 inoculated pigs, but no controls, developed mucoid or mucohaemorrhagic diarrhea (MHD). Characteristic lesions of swine dysentery were observed in large intestine. *"B. hampsonii"* strain 30599 DNA was detected by qPCR in feces of all inoculated pigs for up to 6 days prior to the onset of MHD. The organism was isolated from the feces and colons of pigs demonstrating MHD, but not from controls. *B. intermedia* was isolated from inoculated pigs without MHD, and from 5 of 6 controls. We conclude that "*Brachyspira hampsonii*" clade I strain 30599 is pathogenic and causes mucohaemorrhagic diarrhea and colitis in susceptible pigs. Moreover, the three sampling methodologies performed similarly. GenoTube Livestock, a forensic swab designed to preserve DNA during shipping is a useful tool especially in settings where timely transport of diagnostic samples is challenging.

Keywords: "*Brachyspira hampsonii",* swine dysentery, mucohaemorrhagic, bloody, diarrhea, colitis, pig, porcine

3.2 Background

The first published description of swine dysentery (SD) appeared in 1921, although the causative agent was unknown at the time (Whiting et al., 1921b). In 1971, *Treponema hyodysenteriae* (later renamed *Brachyspira hyodysenteriae*) was identified as the pathogen responsible for the syndrome (Harris et al., 1972; Taylor and Alexander, 1971). Until the early 1990's, SD was considered a major production-limiting disease in North American commercial swine farms. Since the mid-2000's, swine producers and veterinarians have observed the re-emergence of mucohaemorrhagic diarrhea (MHD) and colitis in commercial farms (Harding et al., 2013; Harding et al., 2010a; Harding et al., 2011; Schwartz, 2011). North American diagnostic laboratories observed an increase in the number of cases of *Brachyspira hyodysenteriae* diarrhea submitted, as well as cases associated with "atypical" *Brachyspira* spp. (Clothier et al., 2011). Further characterization of these atypical isolates led to the description of a novel *Brachyspira* species, provisionally named "*Brachyspira hampsonii"*. It has been proposed that this new species is comprised of two different phylogenetic clades, sharing 96% sequence identity in the NADH oxidase (*nox)* gene sequence (Chander et al., 2012).

Mucohaemorrhagic diarrhea and colitis, indistinguishable from SD has been experimentally reproduced in pigs with "*B. hampsonii"* clade II isolates from Canada and the United States (Burrough et al., 2012b; Rubin et al., 2013a). Burrough *et al.* has also reproduced SD experimentally in mice (Burrough et al., 2012a) and in pigs (Burrough et al., 2012b) using strongly β-hemolytic strains initially identified as *B. intermedia* based on PCR (Song and Hampson, 2009), which have since been confirmed to be "*B. hampsonii"* clade I (Mahu et al.,

2014). These data suggests that clade I is pathogenic to pigs, but further studies are required to confirm this, evaluate additional strains and to characterize the disease including patterns of shedding.

The first Canadian diagnosis of *"B. hampsonii"* clade I was in November 2011 from grow-finish pigs with bloody, mucoid diarrhea. This was a unique event, since all previous *"B. hampsonii"* cases diagnosed by our laboratory were of clade II. The isolate recovered from this diagnostic case was designated "30599". Since its first diagnosis in Canada, *"B. hampsonii"* clade I (strain 30599) has been identified, isolated or both, in the absence of other *Brachyspira* spp., in feces or tissues from 39 cases of diarrhea from 15 farms in western Canada. Given the distance between farms and diagnostic laboratories in western Canada, this may be an underestimate of the number of farms affected by the organism. One of the main obstacles to obtaining high quality diagnostic samples and reliable results is the transit time required to ship samples to a diagnostic laboratory (Bonini et al., 2002; Waldmann et al., 2000). For PCR based diagnostics, sample quality may be improved by immediate preservation of target DNA in the sample and controlling the growth of opportunistic organisms. A forensic swab (GenoTube Livestock; Prionics, Switzerland) capable of rapidly drying the sample and providing minimal DNA loss after long-term storage without refrigeration may be a potential tool for aiding the diagnosis of infectious diseases by PCR in biologic samples shipped long distances to diagnostic laboratories.

The objectives of this study were to determine the pathogenicity of "*Brachyspira hampsonii*" clade I (strain 30599) when inoculated in naïve pigs, and to investigate the performance of GenoTube Livestock swabs for *ante mortem* sampling of pigs for PCR-based detection of

"Brachyspira hampsonii" clade I. In order to accomplish these primary objectives, we also developed and validated a PCR assay for the detection and quantification of "*Brachyspira hampsonii*" clade I (strain 30599).

3.3 Methods

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (permit #20110038).

3.3.1 S*ource of "B. hampsonii" strain 30599 inoculum*

"B. hampsonii" clade I strain 30599 was isolated from a pig with mucoid diarrhea in Alberta, Canada in November, 2011. PCR for *B. hyodysenteriae* (Song and Hampson, 2009), *B. pilosicoli* (Rubin et al., 2013a) and *"B. hampsonii"* clade II (Rubin et al., 2013a) performed on case tissues returned negative results. Sections of small and large intestines tested negative for *Salmonella* (culture on brilliant green agar following enrichment with selenite broth), *Lawsonia intracellularis* (PCR) (Jones et al., 1993), and porcine circovirus type 2 (PCV2, immunohistochemistry) (Harding et al., 2008) at the Prairie Diagnostic Service Inc. (PDS), Saskatoon, SK. Culture of colon tissue on selective media resulted in areas of strong β-hemolysis, which were sub-cultured to obtain an isolate. Sequencing of the *nox*, *cpn*60 and 16S rRNA genes from this isolate demonstrated that it was similar to, but phylogenetically distinct from *"B. hampsonii"* clade II. Whole genome sequencing of the isolate was performed (NCBI

BioProject PRJNA188379) and confirmed its affiliation with what has recently been proposed as clade I of *"B. hampsonii"* (Chander et al., 2012).

3.3.2 Experimental inoculation

The experimental inoculation was performed as described previously ("Pure Broth Culture Inoculation" in (Rubin et al., 2013a)). Briefly, 18 five-week old pigs (16 male, 2 inadvertently female) were obtained from a commercial farm in Saskatchewan, Canada. No history of MHD or previous diagnosis of *Brachyspira* spp. associated diarrhea was reported, and fecal samples collected from nursery (n=20) and grow-finish (n=10) pigs prior to the commencement of the study tested negative for *Brachyspira* spp. by culture and species-specific qPCR for *B. hyodysenteriae, B. pilosicoli* and *"B. hampsonii"* (data not shown). Upon arrival at the Animal Care Unit at the University of Saskatchewan, the pigs were allocated randomly to control (CTRL, n=6) and inoculated (INOC, n=12) groups housed in separate rooms with 3 or 2 pigs per pen, respectively. One female pig was assigned to each group. Pigs were acclimated to their new diet and room environment for 7 days prior to inoculation, and were offered *ad libitum* water and commercially prepared, non-medicated, pelleted starter diet (Whole Earth Pig Starter, Federated Cooperative Ltd., Saskatoon, Canada) formulated with less than 15% soybean meal for the duration of the experiment.

Pigs were sedated with 8 mg/kg azaperone IM (Stresnil, Vetoquinol Canada Inc., Lavaltrie, Quebec) then inoculated by gastric tube on three consecutive days (day (D) 0, 1 and 2). INOC received 10 mL of frozen JBS broth containing 6.10×10^8 (D0) 4.14×10^8 (D1) and 4.24×10^8 (D2) genome equivalents of *"B. hampsonii"* strain 30599, followed by 40 mL of PBS (0.1M, pH

7). CTRL received 10 ml of sterile JBS broth followed by 40 mL of PBS. For both groups, feed was removed 16 h prior to inoculation to increase gastric motility.

Feces and rectal swabs were collected on day -8, -5, -2 and 0 prior to inoculation, and daily thereafter until the end of the experiment. Fecal consistency was scored daily as: 0 = formed, normal; 1 = soft, wet cement consistency; 2 = runny or watery diarrhea; 3 = mucoid diarrhea; 4 = mucohaemorrhagic diarrhea. Daily clinical assessment was performed on all pigs. Rectal temperature, responsiveness to external stimuli (e.g. the presence of researcher in the pen) and skin colour were recorded. Body weight was measured on D0 and D8. INOC pigs were humanely euthanized by cranial captive bolt and exsanguination approximately 24 hours after the development of mucoid or mucohaemorrhagic diarrhea. INOC pigs that did not demonstrate MHD were euthanized on D13; CTRL pigs on D14.

3.3.3 Pathological assessments

A complete necropsy was performed and the gastrointestinal tract was completely removed from stomach to rectum, including mesenteric lymph nodes. Special attention was paid to the cecum and spiral colon which was completely linearized and divided into thirds (proximal, apex and distal). The mucosal surfaces of the large intestines were assessed for the presence of characteristic lesions of SD including hyperemia, congestion, edema, necrosis, fibrin and mucus by a single pathologist (SED). A blinded histologic evaluation of colon and cecum was performed by the same pathologist. Tissue samples were fixed in 10% buffered formalin for 24 hours and paraffin embedded. All microsectioned tissues were stained with Hematoxylin-Eosin (H&E) and a serial section of the spiral colon was also Warthin-Faulkner (WF) silver stained.

Lesions in the spiral colon and cecum were scored based on the severity of the inflammation and necrosis: 0 = no lesions; 1 = minimal to mild necrosis of superficial enterocytes with minimal inflammatory infiltrates; 2 = moderate necrosis and attenuation of enterocytes with mild to moderate inflammatory infiltrates; 3 = severe necrosis (erosion or ulceration present) with moderate inflammatory infiltrates predominantly consisting of neutrophils. The presence of *Brachyspira*-like organisms was also scored from 0 to 3 in WF stained sections: 0 = no spirochetes observed; 0.5 = a single gland contained a few spirochetes; 1 = small numbers of spirochetes in multiple glands; 2 = many spirochetes within several glands; 3 = many spirochetes forming thick mats in numerous glands.

3.3.4 Microbiological assessments

Gastrointestinal tissues collected at termination were screened for the presence of other relevant swine enteric pathogens, including *Lawsonia intracellularis* (PCR, ileum), porcine circovirus type 2 (PCV2, immunohistochemistry, ileum and mesenteric lymph node) and *Salmonella* spp. (culture, ileum). All methods were the same as described above. PRRSv IgG antibody and RNA concentration were measured by ELISA (IDEXX PRRS X3, IDEXX Laboratories Inc., Westbrook, ME) and PCR (Tetracore Inc., Rockville, MD) respectively at PDS in sera collected on D0 and at termination.

3.3.5 Quantification of "B. hampsonii" clade I strain 30599

Details on the development and validation of the strain-specific qPCR assay are given in the Results. DNA for PCR was extracted from feces and colon tissue using the QIAmp DNA stool

mini kit (Qiagen Inc., Toronto, ON), and from cultured bacteria and colon tissue using DNEasy blood and tissue kit (Qiagen Inc.). SYBR green real-time qPCR detection of *"B. hampsonii"* strain 30599 was performed on a Bio-Rad MyiQ thermocycler in reactions containing 1× SYBR Green Supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON), 400 nM each primer JH0436 (5'-AAA GTG CCA CAG GCA ATG TA-3') and JH0437 (5'-TGC AAG ATT AGA CGG AGC AA-3')) and 2 µL of template DNA, in a final volume of 25 µL. All qPCRs were run on a plate containing a no-template control and a standard curve composed of target-containing plasmids at concentrations of 10^0 to 10^7 copies/reaction. All reactions were performed in duplicate. Thermocycling parameters included an initial denaturation (95 °C for 3 min), followed by 40 cycles of 95 °C for 15 sec., 63 °C for 15 sec., 72°C for 15 sec., and a final extension at 72°C for 5 min. A dissociation curve was subsequently performed for 81 cycles at 0.5 °C increments from 55 °C to 95 °C. Fluorescent signals were measured every cycle at the end of the annealing step and continuously during the dissociation curve data collection. All resulting data was analyzed using iQ5 Optical System Software (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON).

3.3.6 Brachyspira culture

Isolation of *Brachyspira* spp. was performed on BJ agar from feces and colonic mucosa, as previously described (Rubin et al., 2013a). Presence of *Brachyspira* growth in zones of β-hemolysis was confirmed by dark-field microscopy, and genus-specific PCR targeting the *nox* gene (Rohde et al., 2002) followed by DNA sequencing using the amplification primers.

3.3.7 Comparison of ante mortem sampling techniques

Three *ante mortem* sampling and detection methods were chosen for comparison: rectal swabs (CultureSwab Liquid Stuart, BD Canada, Mississauga, ON) for culture on selective agar, qPCR on DNA extracted from fecal samples (200 mg samples, QIAmp DNA stool mini kit, Qiagen Inc.), and qPCR on DNA extracted from GenoTube swabs. Samples were collected D-8, D-5, D-2 and D0 prior to inoculation, and every other day thereafter to D12 in INOC pigs. Targeted sampling was employed in order to sample pigs with different fecal scores. On each day, samples were simultaneously collected from pigs with no diarrhea (n=2), pigs with "wet-cement" or watery diarrhea (n=2) and pigs with mucoid and mucohaemorrhagic diarrhea (n=2). If one or more category was not observed at the time of collection, pigs with the closest available fecal score on that day were sampled. The pigs from which samples were collected were blocked by pen.

To simulate a field situation where samples would be transported to the diagnostic laboratory over 24 h, culture swabs and feces were refrigerated at 4^{o}C (to mimic shipment with ice packs), while GenoTube swabs were stored at room temperature for 24 h prior to processing. GenoTube swabs were expressed into 1.4 mL lysis buffer (buffer ASL, QIAmp DNA stool mini kit, Qiagen Inc., Toronto, ON) in a 2 mL microcentrifuge tube by pressing the swab head against the inside wall of the tube while twisting. To ensure consistent sample expression, swabs were turned exactly ten times each (5 clockwise and 5 counter clockwise). Following expression, extraction proceeded according to the kit instructions. DNA extracts (2 µL each) from feces and GenoTube

swabs were used as a template for *"B. hampsonii"* clade I specific qPCR analysis. Culture swabs were used to inoculate BJ agar plates.

3.3.8 Statistical analysis

Statistical analysis was performed using SPSS v19.0 (SPSS Inc., Chicago, IL) or Stata v13 (StatCorp, College Station, TX). Group differences in the number of pigs demonstrating MHD (fecal consistency score 4) was compared using a Fisher's exact test. The same test was used to compare the presence or absence of specific gastrointestinal lesions based on gross and histopathological assessments. Continuous variables including average daily gain (ADG) and the concentration of "*B. hampsonii"* strain 30599 DNA detected by qPCR were non-parametrically distributed and compared between groups using a Kruskal-Wallis Analysis of Variance. Spirochete scores in colon were compared with a Mann-Whitney U test. To compare the severity of gross lesions among proximal, apex and distal colon segments, a Wilcoxon matched-pairs signed-rank test was used after assigning negative, mild, moderate and severe lesion scores as 0, 1, 2, 3 respectively. For all analyses, $P < 0.05$ was considered as statistically significant.

3.4 Results

3.4.1 Development of "B. hampsonii" clade I strain 30599 specific SYBR green PCR assay

A strain-specific, SYBR green quantitative real time PCR assay was developed to quantify "*B. hampsonii"* strain 30599 DNA. Primers JH0436 (5'-AAA GTG CCA CAG GCA ATG TA-3')

and JH0437 (5'-TGC AAG ATT AGA CGG AGC AA-3') were designed to target a 176 bp region within a predicted open reading frame in the *"B. hampsonii"* strain 30599 genome encoding a hypothetical protein (Genbank accession WP_008726773). The target region lies within an approximately 15.8 kb region of the genome identified as being unique to strain 30599 in a comparison of the whole genome sequences of clade I strain 30599 (BioProject PRJNA187424) and clade II strain 30446 (BioProject PRJNA169353). The target sequence has no significant sequence identity to *"B. hampsonii"* clade II strain 30446, and is only 90% identical at the nucleotide level to a sequence within the genome of *B. intermedia* PWS/A (ATCC 51140^T). No other significant sequence identities to other *Brachyspira* species were identified.

An optimal annealing temperature of 63 °C was determined, and a linear standard curve was obtained over a range of 10^0 to 10^7 target copies per PCR using a ten-fold dilution series of cloned target amplicon in plasmid pGEM T Easy as template.

Analytical specificity was determined initially by applying the primers to genomic DNA from *B. hyodysenteriae* ATCC 27164^T, *B. pilosicoli* ATCC 51139^T, *B. intermedia* ATCC 51140^T, *B. innocens* ATCC 29796^T, *B. murdochii* ATCC 51284^T, and *"B. hampsonii"* clade II strain 30446. No amplification was detected from any template other than *"B. hampsonii"* clade I strain 30599. Further validation of the analytical specificity of the *"B. hampsonii"* strain 30599 specific qPCR involved its application to fecal samples from previous clinical cases determined to be positive for *"B. hampsonii"* strain 30599 by amplification and sequencing of the *nox* gene using previously published primers (n=25) (Rohde et al., 2002), and fecal samples from healthy pigs

from the source farm that were confirmed negative for *Brachyspira* by the same method (n=30). All (30/30) of the negative samples were found negative by the strain specific qPCR, and 24 of 25 of the positive samples were found positive by the strain specific qPCR, demonstrating significant agreement between the methods.

3.4.2 Acclimation period

Brachyspira culture was performed on all pigs on four days during the pre-inoculation acclimation period. *B. intermedia* (97% *nox* gene sequence identity over 810 bp to *B. intermedia* ATCC 51140^{T}) was isolated from one INOC and one CTRL pig on D-2, and from one INOC on D0. *"B. pulli"* (98% nucleotide identity over 848 bp of *nox* to strain AN304/04) was isolated from one INOC on D-2 and D0. *"B. pulli"* is the provisional name for a spirochete originally identified in birds (Stephens and Hampson, 2001), which has recently also been isolated from pigs (Osorio et al., 2013). One CTRL had watery diarrhea on D-8 and D-7, immediately after arrival in the animal care facility, but *Brachyspira* was not isolated, and normal feces were observed during all other days pre-inoculation.

3.4.3 Clinical observations

One INOC pig presented with tachypnea and dyspnea on D3 and D4, and for welfare reasons was euthanized and necropsied on D5. Necropsy findings indicated aspiration of feed at the tracheal bifurcation. Data from this pig were removed from all analyses.

Post-inoculation fecal scores for INOC pigs are summarized in Figure 3.1. Eight of 11 INOC pigs developed MHD (score 4)(Figure 3.2), and one INOC developed mucoid diarrhea (#22,

score 3). Of the two remaining INOC pigs, one (#19) remained healthy throughout the study, and one (#670) had fecal consistency typical of wet cement (score 1) on four post-inoculation days. To distinguish these from the nine diarrheic INOC pigs, they are referred to as "INOC (without MHD)". No CTRL pig demonstrated mucoid or bloody diarrhea after sham inoculation, although one had fecal consistency typical of wet cement (score 1) on D6 and D9, and one had runny diarrhea (score 2) on D4 and wet cement consistency on D8, but was normal on all other days. Mucohaemorrhagic diarrhea was significantly more frequent in INOC than in CTRL pigs (P=0.002).

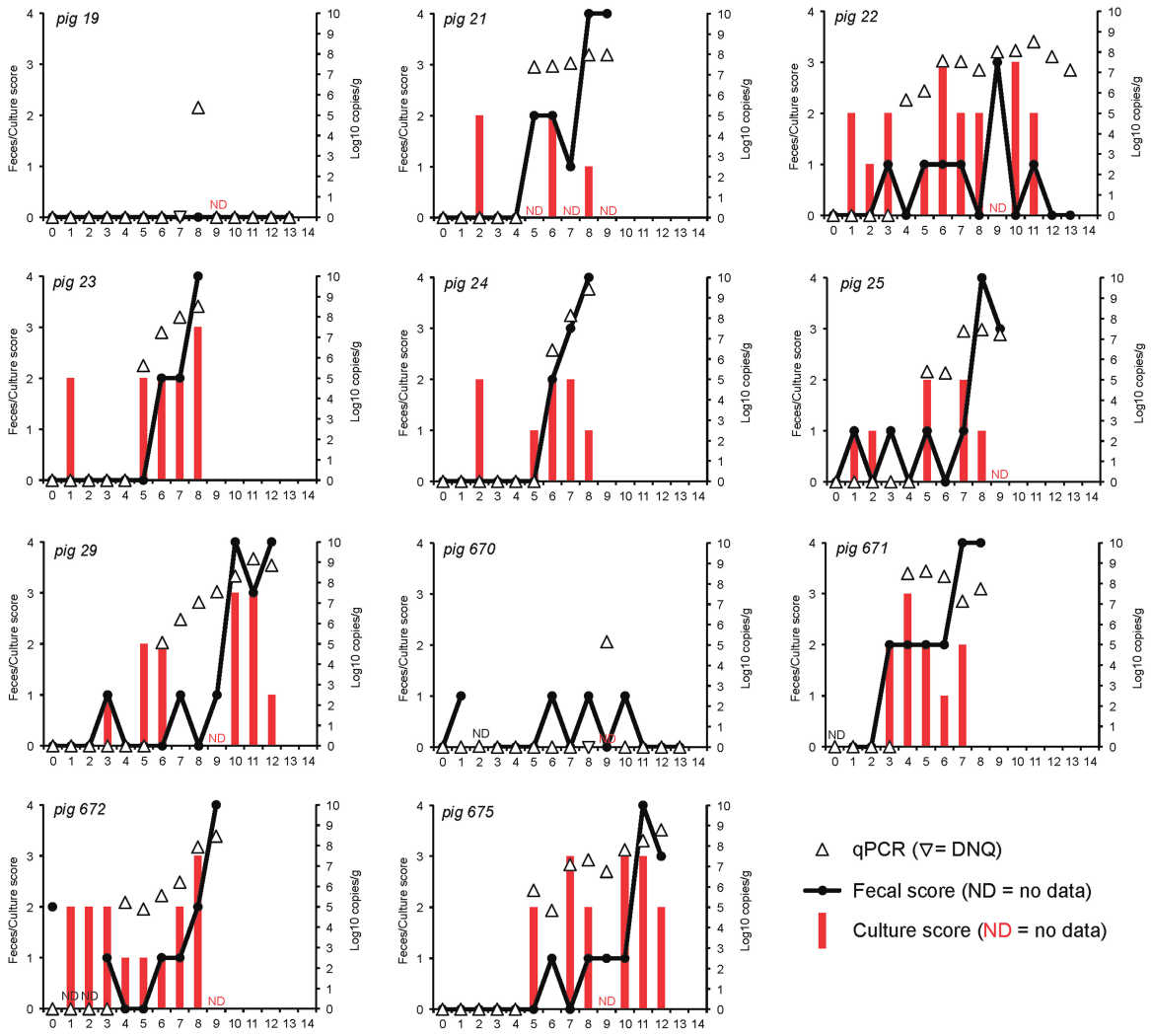


Figure 3.1. Fecal consistency scores, culture results and fecal concentration of *"Brachyspira hampsonii"* strain 30599 following inoculation.

Fecal consistency scores (line, left ordinate: 0 = formed, normal; 1 = soft, wet cement consistency; 2 = runny or watery; 3 = mucoid diarrhea; or 4 = bloody diarrhea), semi-quantitative fecal culture score (red bars, left ordinate: 0 = negative; 1 = less than 10 colonies/1° streak; 2 = less than 10 colonies/2° streak; 3 = less than 10 colonies/3°streak; 4 = less than 10 colonies/4° streak), and strain 30599 DNA concentration in feces (triangles, right ordinate;

upside down triangles indicate DNQ) are shown. Days post-inoculation are shown on the abscissa. ND = no data. Pig IDs are indicated in the upper left corner of each panel.

Figure 3.2. Mucohaemorrhagic diarrhea. Accumulation of loose and runny feces with mucus and frank blood.

Pig #675, 11 days post inoculation, day of first observation of mucohaemorrhagic diarrhea, 7th day of *"B. hampsonii"* strain 30599 shedding, feces culture positive (3+ strongly beta-hemolytic *"B. hampsonii"* strain 30599) on this day, 1.86 × 10^8 strain 30599 genomic equivalents/g. In inoculated pigs the severity of bloody diarrhea ranged from that containing a few flecks of blood to diffusely haemorrhagic with copious blood and mucus as shown.

On D0, one INOC pig (#672) unexpectedly presented with runny diarrhea and fecal samples were submitted for routine aerobic and anaerobic culture to PDS. The sample was negative for *Salmonella* spp. but *E. coli*. was isolated. PCR results indicated that the *E.coli* isolate was positive for virulence factors AIDA-I and STb. Fecal consistency from this pig scored 0 or 1 until D8, then progressed to MHD by D9. The median number of days from first inoculation to the demonstration of mucoid or mucohaemorrhagic diarrhea in INOC was 8, with the shortest and longest incubation periods at 7 and 11 days respectively. The onset of MHD was generally acute with affected pigs progressing from relatively normal feces (score 0 or 1) to MHD within 2.4 days (range 1-4) (Figure 3.1).

Average daily gain between D0 and D8 was numerically decreased in INOC (359 ± 143 g/day) compared to CTRL (413 ± 123 g/day), but did not differ significantly between groups.

Serum from blood collected on D0 and at termination was PRRSv PCR and ELISA negative. Intestinal tissue samples collected at termination tested negative for *Salmonella* sp., *Lawsonia intracellularis* and PCV2 by culture or PCR.

3.4.4 Brachyspira shedding patterns

Brachyspira spp. isolates were recovered from feces from 10 of 11 INOC pigs between D0 and D4. This was likely associated with pass-through of the inoculation dose. Eight of 11 were culture positive for *"B. hampsonii"* strain 30599 solely, two of 11 for *B. intermedia*, one of 11 for *"B. hampsonii"* strain 30599 and *B. pulli* on different days (Figure 3.1). Between D5 and termination, *"B. hampsonii"* strain 30599 was consistently isolated in the feces and from the colon of all INOC pigs that developed MHD (Figure 3.1). *B. intermedia* was isolated from the feces and colon of the two INOC pigs without MHD. *B. intermedia* was also isolated sporadically from the feces of 5 of 6 and from terminal colon in 3 of 6 CTRL pigs. The presence of *B. intermedia* in feces was not associated with diarrhea. Inadvertently, an issue with the culture vessel on D9 resulted in negative cultures from all pigs. Culture positive isolates confirmed as *"B. hampsonii"* strain 30599 had 99-100% sequence identity to the inoculum based on sequencing of the *nox* gene. Any minor differences in sequence are most likely explained by PCR artefact since the Taq DNA polymerase used was not a proofreading enzyme.

Quantifiable levels of *"B. hampsonii"* strain 30599 were detected by qPCR beginning D4 in 11 of 11 INOC pigs (Figure 3.1). "*B. hampsonii"* strain 30559 was not detected in CTRL pigs. In INOC pigs without MHD, quantifiable levels were detected on a single day in each pig (D8 or D9). In INOC pigs with MHD, quantifiable levels of *"B. hampsonii"* strain 30599 were detected over multiple consecutive days preceding euthanasia. On average, quantifiable levels were detected for four days prior to the onset of MHD (range 2 to 6). The average concentration of

"B. hampsonii" strain 30599 across all days in INOC pigs with MHD was 8.05 (log 10 genomic equivalents/gram of feces).

Between D1 and D4, *"B. hampsonii"* strain 30599 was isolated by culture in 15 fecal samples of INOC pigs with MHD. In these same samples, the organism was detected by PCR 13 times. Of these, 3 samples had quantifiable levels of DNA whereas the remaining 10 had detectable but non-quantifiable levels (i.e. beyond the lower limit of the linear portion of the standard curve). From D5 to termination, *"B. hampsonii"* strain 30599 was isolated by culture in 37 fecal samples of INOC pigs with MHD, whereas DNA was detected in the same samples 41 times, always at quantifiable levels. These data indicate that PCR and culture had similar overall detection frequencies.

3.4.5 Pathological findings

A summary of gross pathological findings is presented in Table 3.1. Gross lesions in INOC pigs were confined to the large intestine, cecum and rectum. Cecum and rectum of INOC pigs displayed mild mucosal lesions, characterized as hyperemia associated with mucoid and fibrinous exudation. No lesions were observed in CTRL or in the small intestine of INOC pigs. In the two INOC without MHD, no lesions were observed in the proximal, apex or distal spiral colon; however, mild hyperemia of cecal mucosa was noted in one of these two pigs. Of the nine INOC with MHD, characteristic gross lesions of swine dysentery were observed consistently in spiral colon and cecum (Table 3.1). These lesions ranged in severity from mild, mucosal edema and hyperemia to severe, multifocal to coalescing, mucosal congestion with mucofibrinous exudate and focal areas of necrosis (Figure 3.3). In proximal, apex and distal spiral colon, gross

lesions were significantly more frequent in INOC than in CTRL (P<0.05 for all). In rectum, gross lesions were significantly more frequent in INOC with MHD than in CTRL (P=0.04). Typhlitis was not a remarkable finding in INOC pigs, and lesion frequency did not differ between INOC and CTRL groups in this study. These results are contrary to previous descriptions of infections with *B. hyodysenteriae* or "*B. hampsonii"* clades I and II (Burrough et al., 2012b; Harris et al., 1972; Jensen et al., 2010; Rubin et al., 2013a). In INOC pigs with MHD, gross lesions were significantly more severe in the proximal and apex regions of the colon than in the distal colon ($P < 0.01$).

Table 3.1. Comparison of gross and histopathological lesion frequency in CTRL and INOC groups

Description	CTRL (n=6)	INOC (n=11)	INOC (with MHD) (n=9)	*P* value INOC vs. CTRL	*P* value INOC (with MHD) vs. CTRL
Gross pathology frequency*					
Proximal colon	1/6	8/11	8/9	0.04	0.01
Apex colon	0/6	9/11	9/9	<0.01	<0.001
Distal colon	0/6	7/11	7/9	0.02	<0.01
Cecum	1/6	6/11	5/9	ns	ns
Rectum	0/6	5/11	5/9	0.08	0.04
Histopathologic lesion frequency**					
Colonic inflammation	0/6	9/11	9/9	<0.01	<0.001
Colonic necrosis	0/6	9/11	9/9	<0.01	<0.001
Cecal inflammation	0/6	6/11	6/9	0.04	0.001
Cecal necrosis	0/6	3/11	3/9	ns	0.02
Colonic spirochetes	0/6	8/11	8/9	<0.01	0.001

*Gross lesions include mucosal edema, hyperemia, congestion, with multifocal to diffuse mucofibrinonecrotic exudate

**Histopathological lesions and colonic spirochete scores graded 0 to 3 reflecting negative, mild, moderate and severe inflammation or necrosis. Scores greater than 1 were considered lesional.

Figure 3.3. Mucohaemorrhagic colitis.

Congested mucosal surface of apex region of the spiral colon with thick layer of mucus and fibrin and occasional foci of haemorrhage. Pig #29, 12 days post inoculation, 48 hours post onset of mucohaemorrhagic diarrhea.

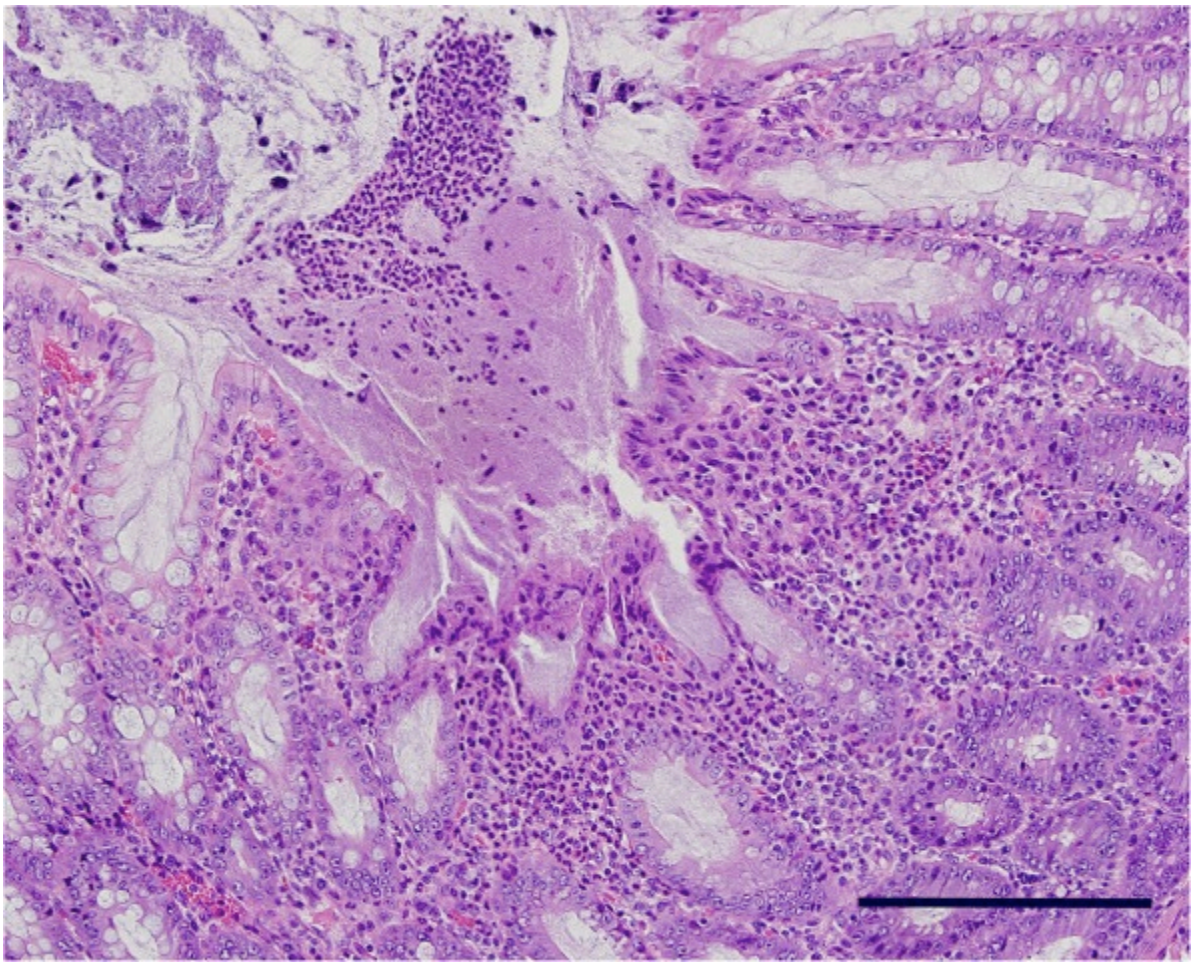


Figure 3.4. *Brachyspira* colitis.

In a section of proximal colon (Pig #671) there is a focal area of epithelial necrosis surrounded in the lamina propria by a mixed inflammatory infiltrate composed primarily of degenerate neutrophils, and covered by a thick mat of mucous containing degenerate neutrophils, necrotic epithelial cells, and bacterial colonies. Hematoxylin and Eosin. Bar = 200 μm.

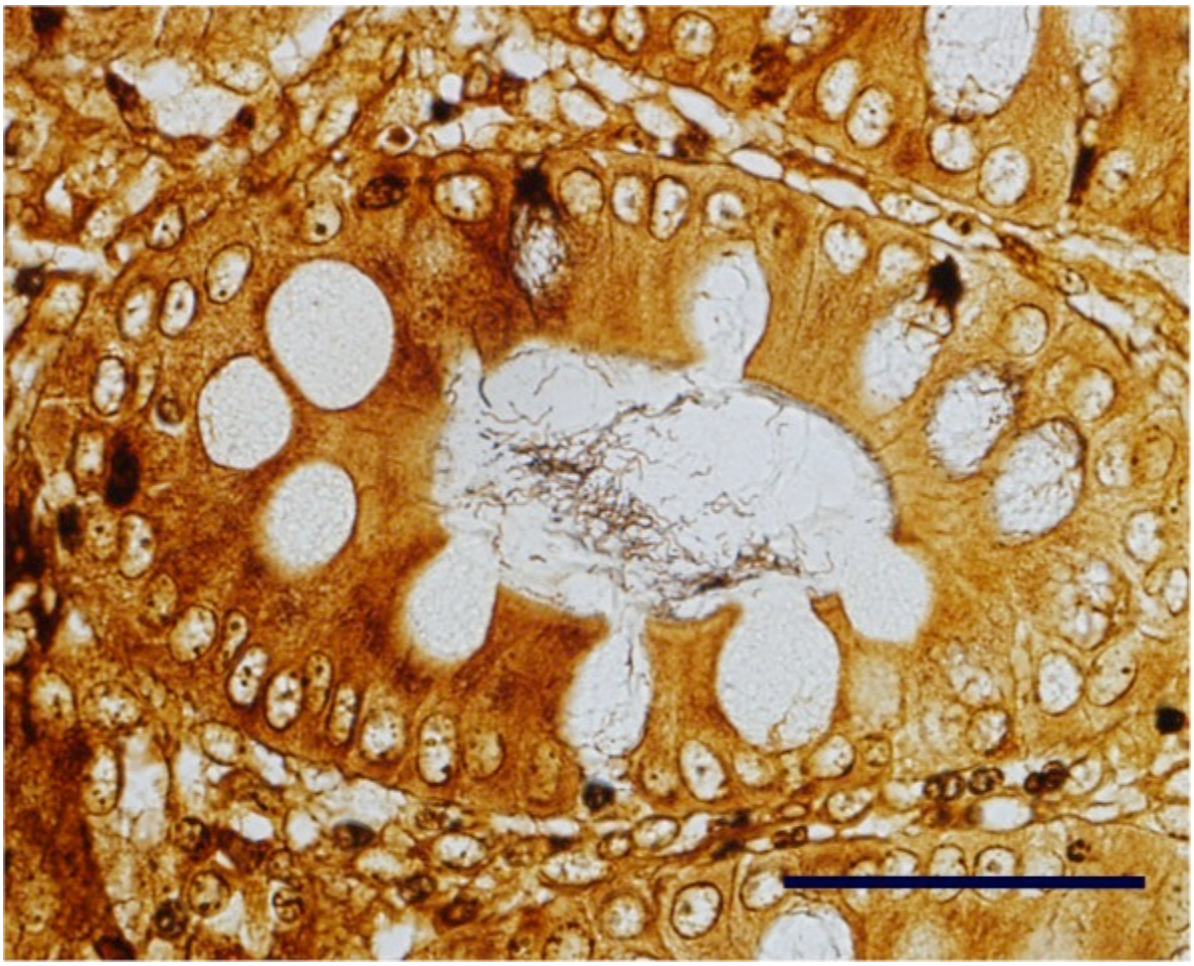


Figure 3.5. *Brachyspira* colitis.

In the same section of proximal colon as Figure 3.4, the tubular mucous-secreting glands contain large numbers of long, thin spiral-shaped bacteria. Warthin-Faulkner. Bar = 100 μm.

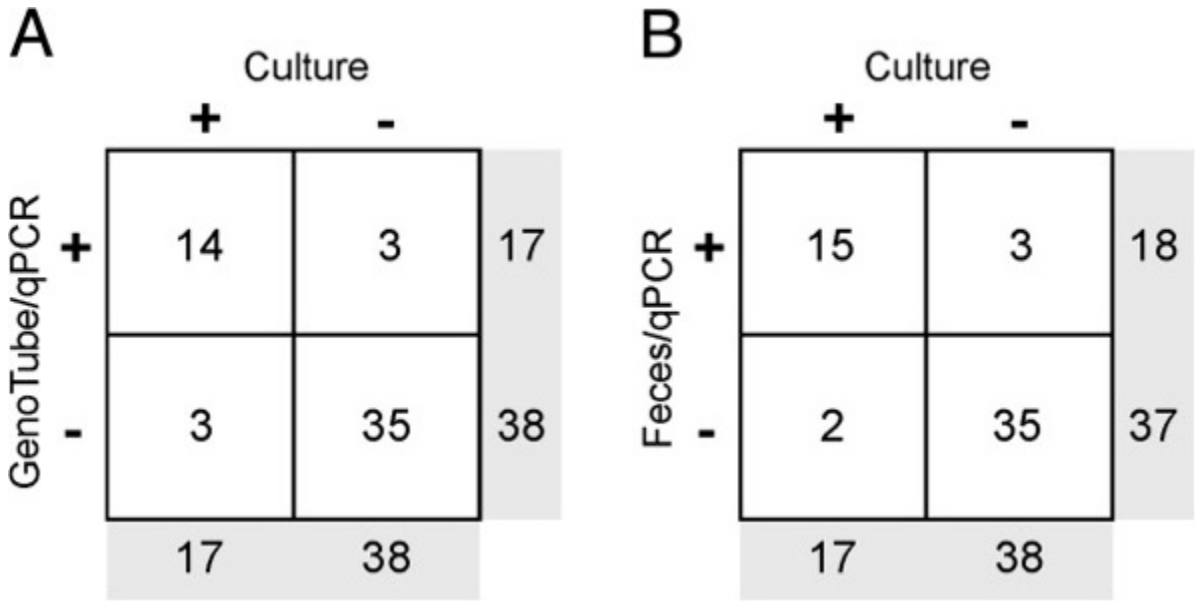


Figure 3.6. Comparison of detection of "*B. hampsonii*" strain 30599 by qPCR on DNA extracts from feces or GenoTube swabs, or culture on selective media.

Contingency tables show the numbers of samples determined to be positive or negative for *"B. hampsonii"* strain 30599 by (A) culture or qPCR on DNA extracts from GenoTube forensic swabs, and (B) culture or qPCR on DNA extracts from fecal samples.

The frequency and severity of histopathologic findings were consistent with clinical signs and macroscopic lesions (Table 3.1). A thick layer of mucus and neutrophils (Figure 3.4) was observed on the colonic mucosa in all but one INOC pig. The nine of eleven pigs with positive cultures had histologic scores of 2 to 3 in the colon. By contrast, five of six CTRL pigs demonstrated mats of Gram-negative rod-shaped bacteria with minimal to no mucus (histologic score of 1). The presence of inflammation and superficial necrosis in the colon and cecum was higher in INOC than CTRL pigs (Table 3.1), as were numbers of spirochetes visualized in colon using silver staining (Table 3.1, Figure 3.5). Inflammation and necrosis were more severe in colon than cecum ($P < 0.01$).

3.4.6 Evaluation of ante mortem sampling techniques

One-hundred and sixty-five samples (55 GenoTube swabs, 55 culture swabs, 55 fecal samples) were collected. Since there were no diarrheic pigs on the majority of sampling days, 78% of samples (43/55) were obtained from pigs with normal feces (score 0 or 1), whereas 9% (5/55) originated from pigs with loose to watery feces (score 2) and 13% (7/55) from pigs with mucoid or mucohaemorrhagic diarrhea (score 3 or 4). Forty-two percent (23/55) of the fecal samples were collected prior to inoculation.

Culture on BJ media followed by sequencing of the *nox* gene was set as the diagnostic gold standard for detection of *"B. hampsonii"* clade I strain 30599. A comparison of detection rate of *"B. hampsonii"* strain 30599 across the three *ante mortem* sampling methodologies is shown in Figure 3.6. Only 12 samples were collected from pigs with runny, mucoid or mucohaemorrhagic diarrhea (score >1). Two of these were negative by culture, two were negative by GenoTube

PCR, and one was negative by PCR of fecal DNA extracts. Both PCR methodologies detected *"B. hampsonii"* strain 30599 in all samples of mucoid and mucohaemorrhagic diarrhea (score 3 or 4). On the other hand, one sample of mucohaemorrhagic diarrhea collected on D8 was negative by culture, but positive by both PCR methodologies. This sample had 5.07×10^7 genome equivalents/g of feces based on qPCR. While this dataset is small, biased towards negative test results, and limited to a single experiment, the results suggest these assays have similar detection capabilities.

3.5 Discussion

The results of this experiment confirm that *"B. hampsonii"* clade I strain 30599 is pathogenic in susceptible pigs, and results in clinical disease indistinguishable from swine dysentery associated with *B. hyodysenteriae* and *"B. hampsonii"* clade II. This finding is relevant for pork producers in Canada and elsewhere whose herds experience mucohaemorrhagic diarrhea. To our knowledge this is the first report of experimental reproduction of disease associated with Canadian strain 30599. Moreover, a previous report from Burrough *et al.* (Burrough et al., 2012b) pre-dated our present understanding based on *nox* sequences, that the strongly β-hemolyic strains of *B. intermedia* (KC35, EB106) used in these experiments were in fact *"B. hampsonii"* clade I. We hope the present research will help to clarify any potential confusion that may exist regarding the pathogenicity of this organism.

A second substantial deliverable of this research is the first description of a novel quantitative PCR assay specific for *"B. hampsonii"* clade I. Based on a discriminating region of the genome,

this SYBR-based PCR assay enables quantification, and does not cross react with other known *Brachyspira* species including *"B. hampsonii"* clade II.

Three *Brachyspira* species are capable of causing bloody diarrhea: *B. hyodysenteriae, "B. hampsonii"* clades I and II, and *"B. suanatina". B. hyodysenteriae* is globally distributed, while *"B. suanatina"* is reported only in Europe. Although *"B. hampsonii"* was discovered and mainly recognized in North America, isolations of clade I in Europe have been reported recently. One report details the isolation from two gilts that were imported from the Czech Republic to Belgium with soft watery feces within dilated large intestines (Mahu et al., 2014). The second account describes the isolation from a group of 17 grower pigs demonstrating mild to moderate diarrhea imported from Belgium to Germany (Rohde et al.). A third study described the first finding of "*B. hampsonii"* from feces of birds, more specifically graylag geese and mallards in northwestern Spain (Martinez-Lobo et al., 2013).

In the present study, the onset of mucoid or mucohaemorrhagic diarrhea in INOC pigs occurred within 7-11 days of inoculation, and was preceded by the fecal shedding of high levels (10^5 to 10^8 genomic equivalents/gram of feces) of *"B. hampsonii"* strain 30599 for up to 6 days. The prevalence of MHD was similar to that observed in the *"B. hampsonii"* clade II strain 30446 experimental reproduction (Rubin et al., 2013a) that used a similar animal model. It must be noted that this animal model does not require the feeding of 100% soybean meal prior to inoculation although a 16-hour period of fasting to enhance gastric motility is performed prior to each inoculation.

Swine dysentery is a multifactorial disease, with clinical expression dependent on individual host factors such as the gut microbiota (Durmic et al., 1998), environmental conditions including diet composition (Hansen et al., 2010; Pluske et al., 1996), and presence of a virulent organism. Interestingly, two challenged pigs did not develop diarrhea and from both, *B. intermedia* was isolated in feces and in colonic tissue collected at termination. *B. intermedia* is generally accepted to be a gut commensal and non-pathogenic to pigs (Fellstrom and Gunnarsson, 1995; Hampson et al., 2006; Komarek et al., 2009). Whether or not it played a role in preventing the development of diarrhea in these pigs is not known, but *B. intermedia* was also isolated in five control pigs, suggesting it was a normal inhabitant of the pigs used in this study. A wide range of *Brachyspira* species can be isolated from healthy pigs from commercial farms (Patterson et al., 2013) and are presumed to contribute to the commensal microbiota. The isolation of species other than that contained in the inoculum should therefore not be unexpected.

One pig (#22) developed a mucoid diarrhea with no evidence of blood prior to recovery over several days. This differed from pigs experimentally inoculated with *"B. hampsonii"* clade II, where 100% of the animals that displayed diarrhea progressed to blood-stained feces within 8 days of inoculation (Rubin et al., 2013a). Field veterinarians, upon submission of diagnostic samples in western Canada, have often communicated variation in clinical presentation, particularly diarrhea associated with *"B. hampsonii"* clade I. We have further studies underway to help improve the understanding of the disease pathogenesis, and potential differences in pathogenic mechanisms associated with the pathogenic *Brachyspira* species.

Inoculated pigs with diarrhea had numerically lower ADG compared to non-challenged controls. However, due to study design limitations, experimental power was low and we could not investigate long-term effects of *"B. hampsonii"* clade I on the ADG of pigs. There are numerous reasons why ADG may be chronically depressed in surviving pigs, including any potential effects of *"B. hampsonii"* on the colonic mucosa and microbiota. These are relevant issues for the swine industry that are certainly worthy of future research.

The performance of qPCR on DNA extracted from GenoTube swabs was found to be comparable to qPCR on DNA extracted from feces, or culture. Rectal swabs are an easy and quick tool, enabling efficient sampling of large numbers of pigs, with minimal stress to the pigs and collectors. Moreover, a large percentage of laboratory errors occur during the pre-analytical phase of diagnosis, prior to samples arriving at the diagnostic laboratory (Bonini et al., 2002; Plebani and Carraro, 1997). This, and the fact that GenoTube swabs preserve DNA eliminating the need for refrigeration, makes it a practical, yet reliable methodology for sampling pigs with *Brachyspira*-associated diarrhea. However, the methodology does have several important limitations including the inability to isolate by culture. In addition, the use of rectal swabs precludes the ability to quantify target DNA in feces. Quantification may be important diagnostically since *"B. hampsonii"* clade I or II can be detected at low concentration ($<10^5$ genomic equivalents/gram) in healthy, non-diarrheic weaner or finisher pigs, whereas levels greater than 10^5 genomic equivalents/gram of feces or tissues (Harding et al., 2011).

3.6 Conclusions

In summary, recent reports describing the emergence of *"Brachyspira hampsonii"* in North America and Europe provide evidence of the importance of this pathogen for the global pork industry. Results of this study confirm the pathogenicity of a Canadian *"B. hampsonii"* clade I strain 30599 and describe the course of disease following experimental challenge. We confirmed that strain 30599 caused mucohaemorrhagic diarrhea indistinguishable from swine dysentery associated with *B. hyodysenteriae* or *"B. hampsonii"* clade II strain 30446. All pigs with bloody diarrhea were both qPCR and culture positive. Gross pathology and histopathology revealed characteristic lesions that were more severe in the proximal and apex regions of the colon, than the distal colon. Testing for other relevant pathogens, including PRRSv, *L. intracellularis*, PCV2 and *Salmonella* sp. were negative. Additionally, we evaluated several *ante mortem* sampling techniques and confirmed that a forensic swab, GenoTube Livestock, designed to preserve DNA during shipping may be a useful tool for diagnostic and surveillance projects, especially in settings where timely transport of diagnostic samples is challenging. Further evaluation of GenoTube swabs in field situations that do not require *Brachyspira* isolation and DNA quantification is warranted.

3.7 Transition statement

We have shown that both "*B. hampsonii*" clades II and I are associated with mucohaemorrhagic diarrhea in naïve pigs. Previously, Koch suggested criteria to help establish the causative relationship between microbe and disease, now referred to as Koch's Postulates (Koch, 1876). Since then, molecular biology has revolutionized the field. More specific and sensitive tests have

been developed, and the application of culture independent methods has shown the large, rich and diverse bacterial population that interacts and resides within its warm-blood hosts. Taking that into account, other authors have suggested an updated version of Koch's postulates (Fredricks and Relman, 1996). The two previous chapters showed that pure culture of both tested strains of *"B. hampsonii"* led to clinical mucohaemorrhagic diarrhea. However, pathogen DNA was detected prior to inoculation. This is in accordance with the revised version of Koch's postulates proposed by Fredricks and Relman where the authors understand that with the application of molecular diagnostic methods, small quantities of pathogen DNA does not reflect colonization nor infection of the host. However, the author acknowledges that it does not exclude the possibility that previous exposure to the pathogen may be sufficient for the development of mucosal immunity. In fact, it was observed in both studies and by other authors that not all pigs inoculated developed diarrhea or colitis, with some animals remaining healthy throughout the studied period. Multiple factors could be associated with this, and none of the above-described studies were designed to clarify that. The large commensal bacterial population present in the colon is known to be protective against other enteric pathogens. Our next goal was to characterize the fecal microbial communities from inoculated and control pigs before and after inoculation and investigate the potential protective properties of the microbiota.

4 Characterization of the fecal microbiota of pigs before and after inoculation with *"Brachyspira hampsonii"*

Citation

Costa MO, Chaban B, Harding JCS, Hill JE (2014) Characterization of the fecal microbiota of pigs before and after inoculation with "*Brachyspira hampsonii*". PLoS ONE 9(8): e106399. doi:10.1371/journal.pone.0106399

Author Contributions

Conceived and designed the experiments: JEH JCSH. Performed the experiments: MOC BC. Analyzed the data: MOC BC JEH. Contributed to the writing of the manuscript: MOC BC JCSH JEH

4.1 Abstract

"*Brachyspira hampsonii*" causes disease indistinguishable from swine dysentery, and the structure of the intestinal microbiome likely plays a role in determining susceptibility of individual pigs to infection and development of clinical disease. The objectives of the current study were to determine if the pre-inoculation fecal microbiota differed between inoculated pigs that did (INOC MH) or did not (INOC non-MH) develop mucohaemorrhagic diarrhea following challenge with *"B. hampsonii*", and to quantify changes in the structure of the microbiome following development of clinical disease. Fecal microbiota profiles were generated based on amplification and sequencing of the *cpn*60 universal target sequence from 89 samples from 18 pigs collected at -8, -5, -3 and 0 days post-inoculation, and at termination. No significant differences in richness, diversity or taxonomic composition distinguished the pre-inoculation microbiomes of INOC MH and INOC non-MH pigs. However, the development of bloody diarrhea in inoculated pigs was associated with perturbation of the microbiota relative to INOC non-MH or sham-inoculated control pigs. Specifically, the fecal microbiota of INOC MH pigs was less dense (fewer total 16S rRNA copies per gram of feces), and had a lower Bacteroidetes:Firmicutes ratio. Further investigation of the potential long-term effects of *Brachyspira* disease on intestinal health and performance is warranted.

4.2 Introduction

Brachyspira associated colitis and mucohaemorrhagic diarrhea has re-emerged as a production limiting disease of pigs in North America. In Canada, this re-emergence has been largely associated with a recently described species of *Brachyspira* for which the name "*Brachyspira hampsonii"* has been proposed (Chander et al., 2012). The association of *"B. hampsonii*" with disease indistinguishable from swine dysentery caused by *B. hyodysenteriae* has been demonstrated in experimental inoculation studies (Burrough et al., 2012b; Rubin et al., 2013a).

The detection of pathogenic species including *"B. hampsonii*" in healthy pigs (Patterson et al., 2013) suggests that exposure alone is insufficient to cause disease, and that extrinsic factors related to the host, pathogen and environment play a significant role in *Brachyspira* pathogenesis. At the individual host level, the development of disease following exposure to *Brachyspira* is influenced by complex interactions between the spirochete and the host intestinal microbiota. Results of numerous early studies following the initial association of *B. hyodysenteriae* with swine dysentery suggested a role for indigenous bacterial populations in *B. hyodysenteriae* infection (Harris et al., 1978; Meyer et al., 1975; Meyer et al., 1974b; Whipp et al., 1979). This relationship has been investigated indirectly in subsequent decades through studies of the influence of diet on *Brachyspira* susceptibility (reviewed by Alvarez-Ordonez et al. (2013)). However, results are contradictory and the microbiological characteristics that define a "susceptible" pig have not been defined. There is also some evidence that *B. hyodysenteriae* infection can result in perturbation of the intestinal microbiota (Leser et al., 2000; Robinson et

al., 1984), which could contribute to the long-term effects on health and performance that may occur in pigs following recovery from dysentery (Compher et al., 1999).

In a recent experimental inoculation study with "*B. hampsonii*" clade II strain 30446 (Rubin et al., 2013a), 8/12 inoculated pigs developed mucohaemorrhagic diarrhea while 4/12 *"B. hampsonii*" inoculated pigs and 0/6 sham-inoculated pigs did not. Pre-existing immunity in the unaffected pigs was deemed unlikely since all pigs in the study were obtained from a high health farm with no history of swine dysentery or previous laboratory diagnosis of *Brachyspira*, and no use of metaphylatic feed or water medication in the grow-finisher stage. In the current study, we characterized the fecal microbiota of these pigs both before inoculation and at termination in order to determine if the composition of the pre-inoculation fecal microbiota differed between inoculated pigs that did or did not develop mucohaemorrhagic diarrhea, and to quantify changes in the structure of the microbiome following development of clinical disease. Although no specific microbiome profile was associated with susceptibility to development of clinical disease following inoculation, the development of bloody diarrhea in inoculated pigs was associated with a reduction in total 16S rRNA copies per gram of feces, and a lower Bacteroidetes:Firmicutes ratio relative to sham-inoculated pigs and those that did not develop clinical disease following inoculation.

4.3 Materials and methods

4.3.1 Ethics statement

Samples used in this study were collected during a previously published experiment that was designed and conducted in accordance with the Canadian Council for Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply (Protocol #20110038).

Fecal samples

All fecal samples utilized in this study were collected during a previously described experimental inoculation trial (Trial 2. Pure Broth Culture Inoculation (Rubin et al., 2013a)). Briefly, pigs were obtained from a high health commercial farm in Saskatchewan with no history of swine dysentery or previous laboratory diagnosis of *Brachyspira*, and no use of metaphylatic feed or water medication in the grow-finisher stage. Farm selection was based on history, and results of screening of 3 and 6 week old pigs for *B. hyodysenteriae, B. pilosicoli* and "*B. hampsonii*". Eighteen pigs were randomly assigned into two groups, inoculated (INOC, n = 12) and control (CTRL, n = 6). Following an 8-day acclimation period, the INOC group was orally inoculated on each of three consecutive days with *"Brachyspira hampsonii"* clade II strain 30446, while CTRL pigs were sham-inoculated with sterile culture media. Fecal consistency was scored daily as: 0 = formed, normal; 1 = soft, wet cement consistency; 2 = runny or watery; 3 = mucoid diarrhea; or 4 = bloody diarrhea. Pigs were euthanized upon development of mucohaemorrhagic diarrhea (6-12 days) or at 14 days post inoculation (dpi) for INOC pigs that did not develop bloody diarrhea,

or 15 dpi for CTRL. Two groups were identified among inoculated pigs: one that developed mucohaemorrhagic diarrhea (INOC MH, n = 8) and another that did not present mucohaemorrhagic diarrhea, although soft feces (maximum fecal score of 1) were occasionally observed in these pigs during the post-inoculation period (INOC non-MH, n = 4). Diarrhea was not observed in any of the CTRL group (0/6). Fecal samples were collected from individual pigs on -8, -5, -3, 0 dpi and on the day of euthanasia (terminal day), and samples were stored at -80 °C until processing.

4.3.2 DNA extraction and microbiota analysis

Total DNA was extracted from 200 mg fecal samples using a commercial kit (QIAmp DNA Stool Mini Kit, Qiagen Inc., Toronto, Ontario). For microbiota profiles, *cpn*60 universal target PCR was performed as previously described (Schellenberg et al., 2011) using multiplex-identifier (MID) tagged primers to facilitate sample pooling prior to sequencing. Amplicon libraries were sequenced in pools of 16 MID tagged libraries on a GS Junior instrument according to the manufacturer's instructions (Roche, Bradford, Connecticut).

A quantitative PCR assay targeting the 16S rRNA gene was performed on all samples, using the primers from Lee et al. (1996) and the protocol of Chaban et al. (2010) to estimate total bacterial content.

4.3.3 Sequence data processing and assembly

Raw sequence data were initially processed using default on-rig procedures (Roche, Branford, Connecticut). The resulting SFF files were de-multiplexed and used as input for the mPUMA

pipeline (Links et al., 2013) using Trinity (Grabherr et al., 2011) for assembly of operational taxonomic units (OTU) and BowTie 2 (Langmead and Salzberg, 2012) for read mapping and calculation of OTU abundance. OTU sequences were compared to the cpnDB reference database (cpnDB_nr version 20130321)(Hill et al., 2004) using a combination of BLAST and the Smith-Waterman algorithm (watered-BLAST)(Schellenberg et al., 2011) to identify the most similar reference sequences and their taxonomic lineages. OTU sequences of at least 150 bp and with at least 55% nucleotide sequence identity to a reference database sequence were retained for further analysis.

4.3.4 Statistical analysis

Ecological metrics for richness (Chao 1) and diversity (Shannon) were calculated using Mothur (Schloss et al., 2009). Prior to calculation of richness and diversity, all libraries were subsampled to 1000 reads to avoid interpretation errors due to different sampling depths (Gihring et al., 2012). Comparisons of ecological metrics, quantitative PCR values and phylum level abundance data was performed using IBM SPSS version 19.0 (IBM Inc., Chicago, Illinois). Specific tests employed for each comparison are described in the context of the results presented below.

Microbial community profiles generated from *cpn*60 amplicon sequence data were compared using Quantitative Insights into Microbial Ecology (QIIME) v1.8.0 using total read counts for all assembled OTU sequences as input. Beta diversity (ecological distance) was calculated using Bray-Curtis dissimilarity. Average pairwise dissimilarities from 100 bootstrapped datasets at 1000 reads per sample were used to generate UPGMA dendrograms within QIIME.

4.4 Results and Discussion

Environmental and host-associated factors, such as temperature fluctuations, transport, mixing of pigs and other stressful management procedures, diet, and the host gut microbiota are thought to influence the pathogenicity of *Brachyspira* (Alvarez-Ordonez et al., 2013). The indigenous microbiota may exclude pathogens, or limit pathogen growth through competition for resources or production of anti-microbial compounds. In this study, we investigated if fecal microbiota composition differs between pigs that developed mucohaemorrhagic diarrhea following inoculation with *"B. hampsonii"* and those that did not, and if infection leading to disease development disturbed the indigenous microbiota structure. The controlled conditions of an experimental inoculation trial provided an excellent opportunity to investigate these questions since many of the environmental variables that would be encountered in a swine production setting were reduced or eliminated.

Microbiota profiles based on the *cpn*60 universal target sequence were generated for 89 samples from 18 pigs (no sample was available for Pig 683, -8 dpi) resulting in 1,041 to 29,520 sequence reads per sample (median 5,231). Sequence data from the study has been deposited to the NCBI Sequence Read Archive (BioProject PRJNA242423). This sequencing depth provided good coverage of the samples, as indicated by all samples having Good's coverage values ≥ 0.91 (average 0.98 ± 0.02). Following raw data assembly and removal of non-target sequences, 1,141 OTU sequences were available for analysis, with 180 OTU detected in at least half of the samples. Comparison of the OTU sequences to cpnDB_nr (a curated database of reference

sequences, primarily from bacterial type strains) resulted in the identification of 330 different nearest neighbour reference sequences. Nucleotide sequence identity of OTU sequences to reference sequences ranged from 55-100% (median 77%)(Table S1). Bacteroidetes and Firmicutes were the dominant phyla in the fecal microbiota of all pigs across all days (accounting for 93.2 ± 5.2 % of the sequence reads in each microbiome profile), with smaller proportions of Proteobacteria and Actinobacteria; consistent with previous descriptions of the swine fecal microbiota based on either the 16S rRNA or *cpn*60 targets (Hill et al., 2005; Kim et al., 2011).

To determine if pre-inoculation fecal microbiota composition was related to the development of mucohaemorrhagic diarrhea following challenge with "*B. hampsonii*", richness and diversity, total 16S rRNA content, and *cpn*60 based microbial profiles were examined from -8, -5, -3 and 0 dpi samples from INOC MH and INOC non-MH pigs. CTRL samples were excluded from this analysis since their susceptibility to developing clinical disease upon inoculation with *"B. hampsonii"* was not known. No significant differences in richness (Chao1) or diversity (Shannon index) were observed between groups on any sampling day (Figure 4.1). The average total bacterial load in pre-inoculation samples estimated by 16S rRNA gene copies was $\log_{10}$ 11.2 ± 0.6 per gram of feces. A marginally significant difference in 16S rRNA copies per gram was detected at 0 dpi between non-MH ($\log_{10}$ 10.8 ± 0.4 copies/g) and MH pigs ($\log_{10}$ 11.3 ± 0.4 copies/g)(Kruskal-Wallis, P = 0.04)(Figure 4.2). The significance of this observation is difficult to assess and should not be over-stated since the difference was only detectable on one of the four sampling days and may reflect daily fluctuations in total bacteria numbers. Graphical analysis of the phylum level microbiota profiles illustrated the similarity of composition among

pre-inoculation samples (Figure 4.3), and when proportions of the major phyla were compared among the groups on each sampling day, the only difference detected was that the proportion of Proteobacteria in INOC non-MH pigs was significantly greater than in INOC MH pigs at -8 dpi (Kruskal-Wallis, $P = 0.01$)(Figure 4.4). The difference in proportional abundance of Proteobacteria could be due to factors affecting individual animals prior to arrival at the study site, and it seems unlikely that it was biologically significant since it was not detected on any of the subsequent sample days leading up to inoculation. The overall similarity of pre-inoculation microbiota composition between INOC MH and INOC non-MH groups was further illustrated by the lack of clustering of groups based on Bray-Curtis dissimilarity values for day 0 profiles (Figure 4.5).

Inoculation with *"B. hampsonii"* resulted in 8/12 of INOC pigs developing mucohaemorrhagic diarrhea. Terminal restriction fragment length polymorphism analysis has been used previously to detect a "destabilization" of the microbiota of pigs inoculated with *B. hyodysenteriae,* but due to limitations of the technique, the authors lacked the ability to characterize the change in terms of taxa involved (Leser et al., 2000). To visualize and quantify any changes to the fecal microbiota of INOC MH pigs in this study, richness and diversity, total 16S rRNA and *cpn*60 microbiota profiles for CTRL, INOC MH and INOC non-MH groups were compared within and between groups at 0 dpi (immediately prior to inoculation) and terminal day (6-12 dpi for INOC MH, 14 dpi for INOC non-MH, and 15 dpi for CTRL).

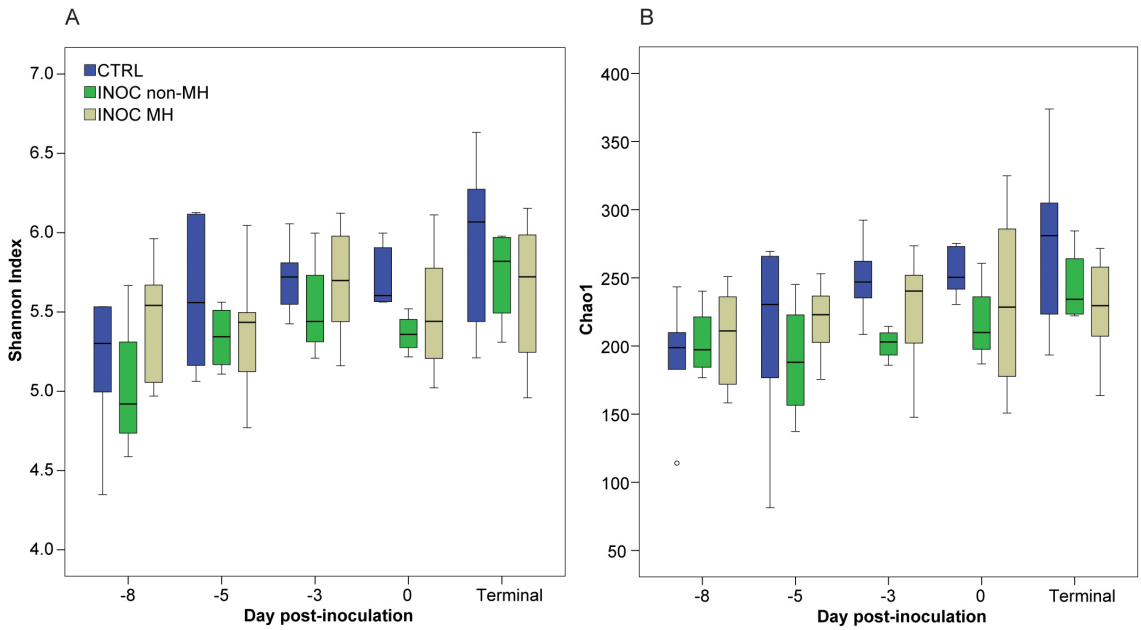


Figure 4.1. Diversity and richness indexes.

(A) Shannon diversity and (B) Chao 1 estimate of richness for CTRL, INOC non-MH and INOC MH groups on all sample days.

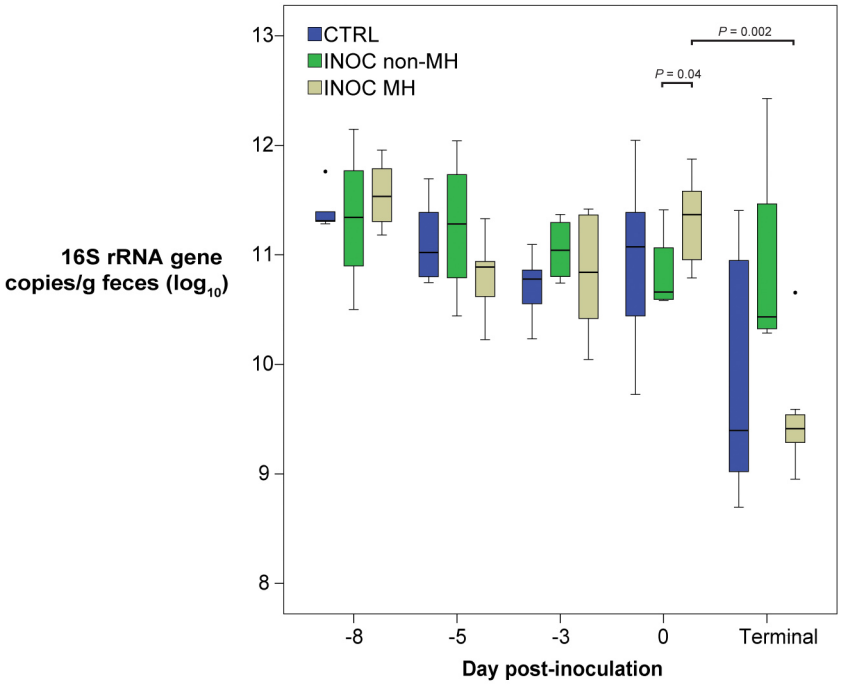


Figure 4.2. Total bacterial load from fecal samples.

Total bacterial 16S rRNA copy numbers per gram of feces in pre- and post-inoculation fecal samples. Significant differences are indicated with *P* value (Kruskal-Wallis, $P < 0.05$).

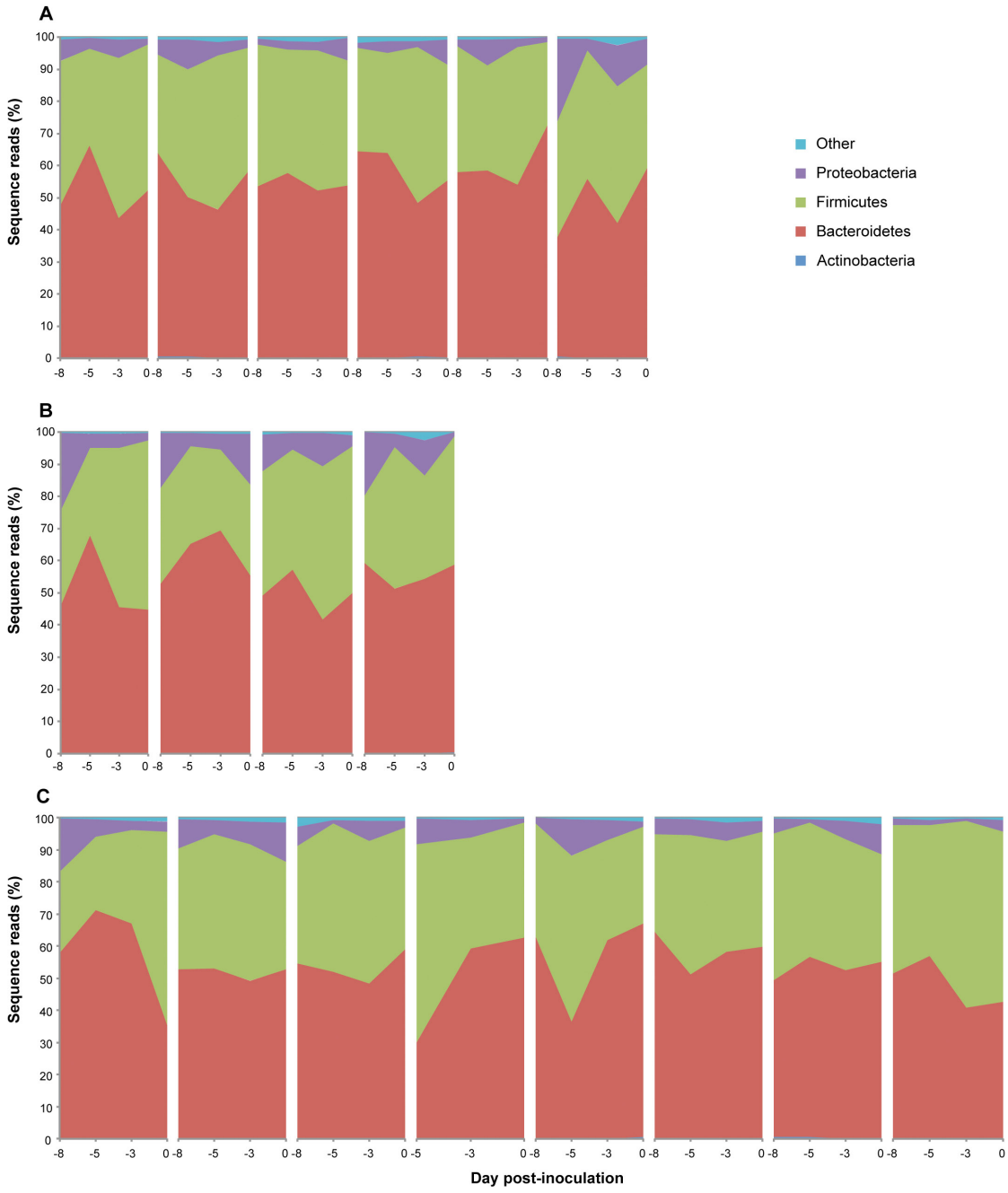


Figure 4.3. Pre-inoculation fecal microbiome profiles.

Pre-inoculation fecal microbiome profiles for (A) CTRL, (B) INOC non-MH and (C) INOC MH. Data is shown as proportional abundance of sequence reads assigned to major phyla.

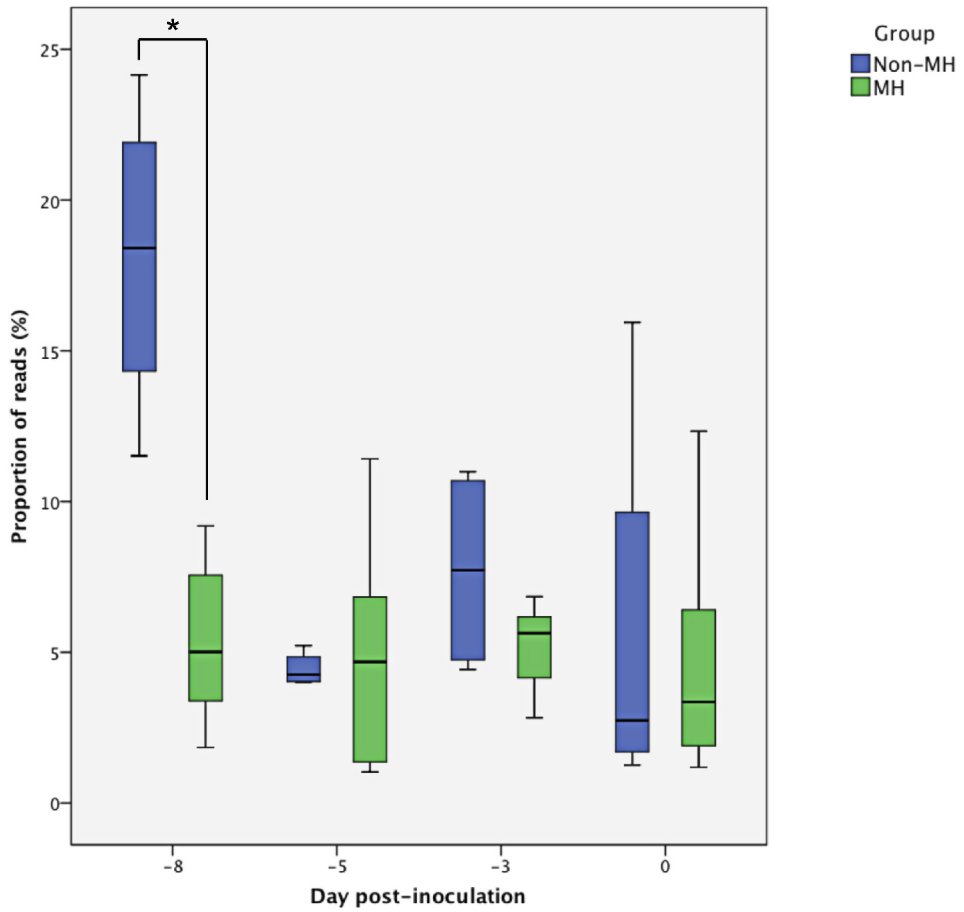
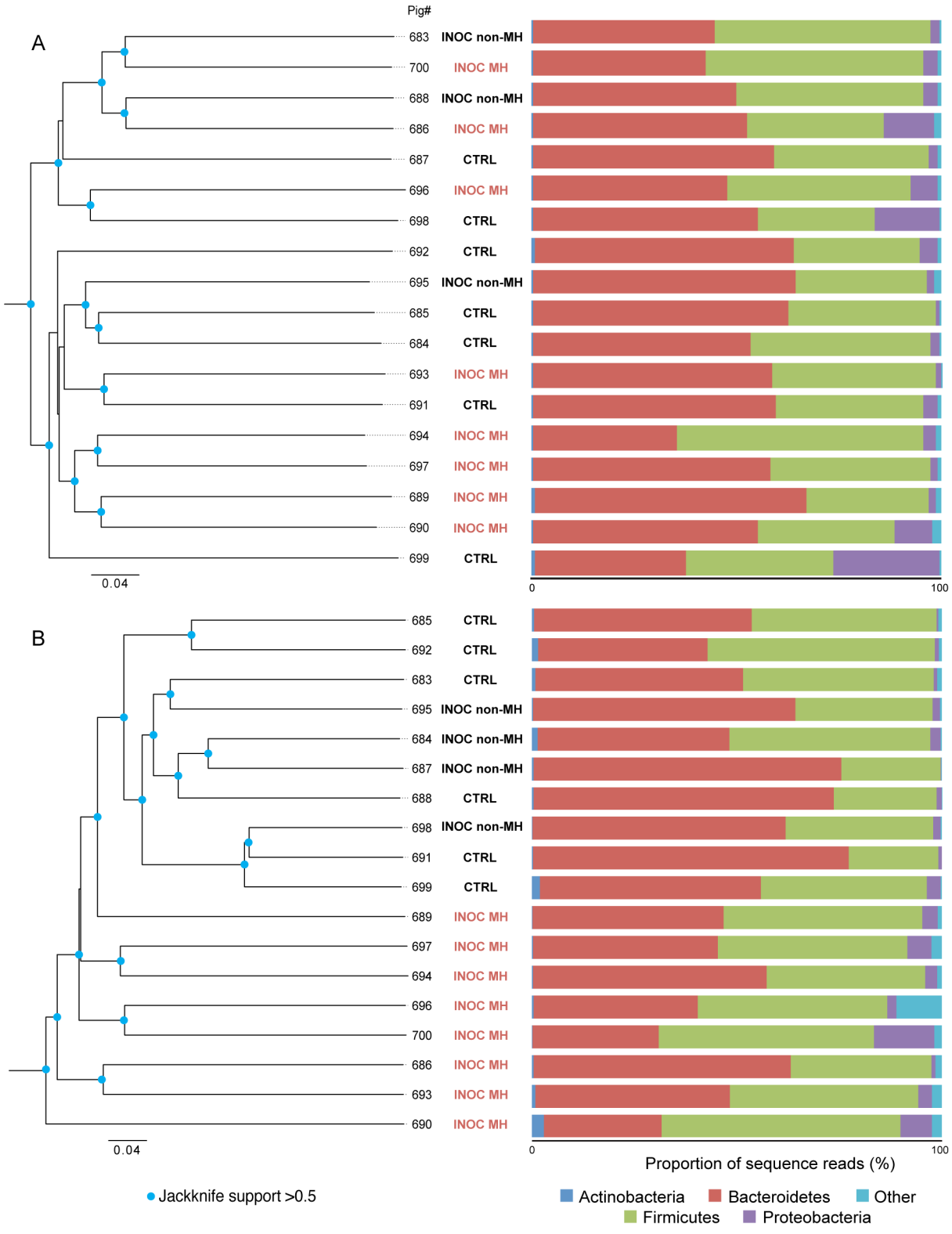


Figure 4.4. Proteobacteria proportion between groups.

Proportion of sequence reads identified as Proteobacteria detected in pre-inoculation fecal samples from MH (n=8) and non-MH (n=4) pigs. A significant difference between the two groups was observed at dat -8 p.i. (Kruskal Wallis, $P = 0.01$).


A
Pig#
683 INOC non-MH
700 INOC MH
688 INOC non-MH
686 INOC MH
687 CTRL
696 INOC MH
698 CTRL
692 CTRL
695 INOC non-MH
685 CTRL
684 CTRL
693 INOC MH
691 CTRL
694 INOC MH
697 INOC MH
689 INOC MH
690 INOC MH
699 CTRL
0.04
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100
B
685 CTRL
692 CTRL
683 CTRL
695 INOC non-MH
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687 INOC non-MH
688 CTRL
698 INOC non-MH
691 CTRL
699 CTRL
689 INOC MH
697 INOC MH
694 INOC MH
696 INOC MH
700 INOC MH
686 INOC MH
693 INOC MH
690 INOC MH
0.04
0
100
Proportion of sequence reads (%)
Jackknife support >0.5
Actinobacteria
Bacteroidetes
Other
Firmicutes
Proteobacteria

Figure 4.5. Microbiota profiles.

Microbiota profile clustering based on Bray-Curtis dissimilarity calculated from abundance of 1141 OTU sequences (dendrogram, left panel), and proportional abundance of major phyla (stacked bar charts, right panel) in each microbiome. (A) 0 dpi, (B) terminal day.

At termination, total 16S rRNA copies per gram of feces were lower for INOC MH (log_{10} 9.3 ± 0.9 copies/g feces) than either INOC non-MH (log_{10} 10.9 ± 1.0) or CTRL (log_{10} 9.8 ± 1.1), due to the significant drop in total 16S rRNA content between pre-inoculation and terminal day in the INOC MH group (Kruskal-Wallis, $P = 0.004$). This was not unexpected since diarrhea results in increased luminal influx of water, decreasing fecal consistency and increasing the number of bowel movements, leading to dilution and washing out of the luminal microbiota and organisms weakly adhered to the surface of the mucosa. Despite the change in overall density of the microbiota, no significant differences or changes in richness or diversity were detected, suggesting that the decrease in dilution effect was general, affecting the entire population (Figure 4.2).

Figure 4.5 shows the relationships of *cpn*60 microbiome profiles of the three groups at 0 dpi and on terminal day based on Bray-Curtis dissimilarity values calculated from abundance of 1141 OTU sequences. Prior to inoculation, the profiles do not cluster according to group (Figure 4.1A). However, terminal day samples from the INOC MH group cluster separately from the others with good jackknife support (Figure 4.1B). Further investigation of the proportional abundance of the major phyla in terminal day samples indicated that the INOC MH group had a lower Bacteroidetes:Firmicutes ratio than the CTRL and INOC non-MH together (Paired sample t-test, $P = 0.036$)(Table 4.1). A lower Bacteroidetes:Firmicutes ratio has also been observed in diarrheic relative to non-diarrheic dogs, regardless of the cause of diarrhea (Chaban et al., 2012), suggesting that the environmental changes in the colon brought about by diarrhea may result in an environment more suitable for survival and growth of Firmicutes than for Bacteroidetes. Examination of differences in the abundances of individual OTU sequences between groups did

not result in the identification of any significant differences, suggesting that the decrease in Bacteroidetes:Firmicutes ratio was the result of a phylum level effect, rather than a change in abundance of any particular species. Robinson et al. (1984) reported a shift in the intestinal epithelial microbiota of dysenteric pigs infected with *B. hyodysenteriae* from Gram positive to Gram negative bacteria, which is not consistent with our observation of a relative increase in Firmicutes (largely Gram positive) in *"B. hampsonii"* affected pigs. However, Robinson et al. used culture-based identification and focused on bacterial populations adherent to the colon epithelium, so direct comparisons with the current study are not appropriate, since the composition of the fecal microbial community is unlikely to be representative of the specialized microbial community closely adhered to the colon epithelium. In humans, the relatively low Bacteroidetes:Firmicutes ratio associated with obesity is thought to contribute to increased risk of *Clostridium difficile* infection in hospital patients with high body mass index (Bishara et al., 2013). Whether a decreased Bacteroidetes:Firmicutes ratio similarly increases risk of infection with other enteric pathogens in pigs remains to be investigated.

Table 4.1. Bacteroidetes and Firmicutes in the fecal microbiota of inoculated pigs at termination.

	CTRL + INOC non-MH (n=8)	INOC MH (n=8)
% Bacteroidetes	59.7 ± 25.6	44.9 ± 11.8
% Firmicutes	37.8 ± 23.3	46.3 ± 7.5
Bacteroidetes:Firmicutes ratio	1.85 ± 0.99[a]	1.03 ± 0.44[b]

[a,b]Superscript letters within a row indicate significant differences.

"B. hampsonii" clade II strain 30446 was detected by species-specific qPCR at levels of $\log_{10}$ 6 to $\log_{10}$ 8 copies/g feces in INOC MH pigs with bloody diarrhea (fecal score = 4)(Rubin et al., 2013a). However, in the current study we identified an OTU corresponding to *"B. hampsonii"* in the microbiota libraries of only 2/8 of the INOC MH pigs (Pig 690 and 694, which had $\log_{10}$ 6.88 and $\log_{10}$ 7.81 copies/g *"B. hampsonii"* by qPCR), and it was detected at low abundance in these libraries (1 and 9 reads, respectively). The low rate of detection of *"B. hampsonii"* in the microbiota libraries is perhaps not surprising given the sequencing depth ($\log_{10}$ 3-4 reads per sample) and that the levels of *"B. hampsonii"* detected by qPCR in these samples corresponded to on the order of 0.01% of total estimated bacterial population (based on 16S rRNA copy number). It is perhaps a testament to the virulence and adaptation of *"B. hampsonii"* that such dramatic clinical effects can result from the presence of a relatively small pathogen population.

Although we did not identify a particular fecal microbiome profile associated with development of mucohaemorrhagic diarrhea following inoculation with *"B. hampsonii*", our results demonstrate that pigs that developed severe clinical disease were distinguished from both inoculated pigs that did not develop clinical disease and sham-inoculated controls by a decrease in the Bacteroidetes:Firmicutes ratio of the fecal microbiota. Larger, prospective studies of experimentally or naturally exposed pigs would be beneficial for detecting more subtle changes in the fecal microbiota and the identification of microbiota profiles associated with susceptibility, but these types of studies are currently prohibitive both logistically and financially. The current study was designed to detect differences in the taxonomic composition of the fecal microbiome.

Future studies employing metagenomics and metabolomics may reveal important functional differences between the pre- and post-inoculation communities.

In the current study, significant phylum level shifts associated with *"B. hampsonii"* bloody diarrhea were apparent. These initial observations of the effects of an economically significant pathogen on its host lead to hypotheses about the potential long-term effects of these changes in the composition of the microbiota, and their possible contribution to poor performance in pigs recovered from *Brachyspira* disease.

Supplemental Information

Table S1. OTU table with read counts and nearest neighbours.

Table S1 cannot be reproduced in the thesis format due to its large size. The complete table is available from the publisher's webpage:

http://journals.plos.org/plosone/article/asset?unique&id=info:doi/10.1371/journal.pone.0106399.s005

4.5 Transition statement

Analysis of the microbiota of pigs resistant to infection by "*B. hampsonii*" did not show any particular profile associated with resistance. Other host-associated factors may play a role in resistance to infection by the spirochaete. In fact, the pathophysiology associated with the early infection by "*B. hampsonii*" is not completely understood. *In vivo* models present a limitation regarding sample collection times, as sampling at the time the spirochaetes infect the colon is challenging. Thus, we proposed to develop an *in vitro* organ culture of porcine colon, which best replicates the host and site of target for "*B. hampsonii*". This model allows proper controlling of infection periods, enabling the study of the early host-pathogen interactions.

5 Development and evaluation of a porcine colon organ culture technique

Matheus O. Costa, John C. S. Harding and Janet E. Hill

Author Contributions

Conceived and designed the experiments: MOC, JCSH and JEH. Performed the experiments: MOC. Analyzed the data: MOC, JCSH and JEH. Contributed reagents/materials/analysis tools: JCSH and JEH. Wrote the paper: MOC, JEH. Primers for porcine IL-8, TNF-α and IFN-γ qPCR were designed by Cole Enns and generously provided by Dr. Matthew Loewen (Department of Veterinary Biomedical Sciences, University of Saskatchewan).

5.1 Abstract

A complex assemblage of specialized tissues that interact in numerous ways forms the intestinal mucosa. *In vitro* cell culture models are generally focused on recreating a specific characteristic of this organ, and do not account for the many interactions between the different tissues. *In vitro* organ culture (IVOC) methods offer a way to overcome these limitations. The objective of this study was to determine the feasibility and optimal conditions for *in vitro* culture of swine colonic mucosa for use as an enteric pathogen infection model. Explants from crossbred commercial pigs (n=12), aged 5 to 10 weeks were used to test the impact of pig age at euthanasia, colon segment, preparation time before culture, antibiotic drugs and culture environment on explant survivability. Explants (n=168) were cultured for up to 5 days, incubated at 37 °C, in a 95% O_2 / 5% CO_2 atmosphere. Explants were fixed in formalin every 24 hours. A second experiment evaluated explants (n=208) from 13 pigs sampled at day 0 and 5 of culture (10% formalin, n=2, and RNAlater, n=4, from each pig at each time-point). Explants were analyzed by H&E staining (both experiments), Warthin-Faulker staining, Ki-67 immunostaining and expression of IL-1α, IL-8, TNF-α and IFN-γ and e-cadherin (qPCR, experiment 2 only). Our data demonstrate that explants were viable after 5 days of culture, with preservation of normal colon tissue architecture. Best possible explants were obtained from the distal colon of pigs, processed immediately after euthanasia, kept in culture media supplemented with a mix of antibiotics and antifungals on a liquid-tissue-air interface. We also observed that age of the pig may be a significant factor, but this requires further investigation since our study design did not allow us to separate age from herd of origin.

5.2 Background

Infection models enable the reproduction of key events of disease processes in a controlled environment. *In vivo* swine infectious disease models are costly, requiring extensive logistics for animal care. In addition, sampling at stages before clinical disease is apparent becomes a challenge, yielding non-optimal samples. *In vitro* cell culture models are generally focused on recreating a specific characteristic of an organ. Cell lines are often limited to a clonal cell population or primary cells that cannot reproduce the original *in vivo* tissue architecture, consequently eliciting a weak pathogen virulence response.

Methods for *in vitro* culture of organ explants (*in vitro* organ culture, IVOC) have been developed as alternatives to overcome some of the limitations of live animal models and cell culture, while still having the advantages of a controlled environment (Deschner et al., 1963; White, 2001). Explants preserve important host characteristics such as different cell types and tissue architecture. In the particular case of the colon, crypts of Lieberkuhn are an important host-pathogen interaction sites, as illustrated in descriptions of *Brachyspira hyodysenteriae* and *Shigella flexneri* infection (Arena et al., 2015; Jacobson et al., 2004). Thus studies targeting colonic pathogens require the presence of crypts to properly model the host. Previous studies have reported *ex vivo* culture of colon tissue from adult human, mouse and rat for periods greater than 24 hours (Autrup et al., 1978a; Browning and Trier, 1969; Dame et al., 2011; Kesisoglou et al., 2006; Shamsuddin et al., 1978).

Culture of swine gastrointestinal tissue culture has been attempted with mixed success. Small intestine is the most frequent organ of choice, since it is the target site for important enteric

pathogens. Previously studied pathogens using porcine small intestine explants include *E. coli* and *Salmonella* spp. (Best et al., 2006; Collins et al., 2010). These studies reported IVOC culture periods ranging from 30 minutes to 6 hours. Colon explants from pigs have also been cultured, but for no longer than one hour (Collins et al., 2010; Danielsen et al., 1982; Kik et al., 1991). An important observation made in these previous studies is that colonic explants exhibit high metabolic activity, which requires supplementation with glutamine. In addition, explants need to be exposed to air in order to supply cells with oxygen, as well as to control media acidification. In order to do that, culture on a rocker platform or in air-liquid interface is required (Abud et al., 2005; Autrup et al., 1978b; Fletcher et al., 2006).

Ex vivo culture of organs for extended periods of time bring important benefits to investigators. It allows for a greater diversity in experimental designs and makes the model more versatile, e.g. it is fit for studies in infectious diseases, nutrition, pharmacology and toxicology. The objective of the current study was to determine optimal conditions for *in vitro* culture of swine colonic mucosa for use as an infectious disease model.

5.3 Materials and methods

The first set of experiments was performed to investigate factors associated with tissue handling that affected survivability of colon tissue explants *in vitro*. The second experiment focused on evaluating the results of application of a protocol developed based on the findings in the first experiment.

5.3.1 Ethics Statement

Experiments were designed and conducted in accordance with the Canadian Council for Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply (Protocol 20130034).

5.3.2 Experiment 1 – Exploring factors affecting explant survival

5.3.2.1 Pigs and animal care

Nine commercial crossbred male pigs at 4 weeks of age were obtained from a high health, PRSS-free commercial farm (Herd 1) and three, 5 week old crossbred male pigs were obtained from a high health research herd (Herd 2). Both herds were in Saskatchewan, Canada. Pigs were housed at the Animal Care Unit, University of Saskatchewan, in 4' x 6' pens floored with rubber mats with two pigs per pen. Pigs were acclimatized to the new environment for a minimum of 7 days, and fed a commercial, non-medicated pelleted starter diet ad libitum. During the acclimation and experimental period pigs were monitored daily for clinical signs of gastrointestinal disease and other abnormalities.

5.3.2.2 Sample collection

Pigs were humanely euthanized by exsanguination after captive-bolt concussion. Immediately after euthanasia, the abdominal cavity was exposed by an incision along the linea alba, from the sternum to the inguinal region. The rectum and the distal oesophagus were ligated to prevent contamination, and the gastrointestinal tract was removed and inspected for any gross lesions. The spiral colon was separated from the ileum, and 10 cm segments of colon were harvested.

Colon loops were placed in 200 mL of Hank's Balanced Salt Solution (HBSS; Gibco Canada Inc., Mississauga, ON, Canada) supplemented with 1.5 mM calcium ($CaCl_2$, Fisher Scientific Ltd, Nepean, ON) and transported to a biosafety cabinet for processing. Tissue was either processed immediately or held in cold HBSS for 2 or 4 hours (Table 5.1)

5.3.2.3 Colon explant culture

All tissue manipulations were performed on a refrigerated surface. Loops were opened along the mesenteric border and the lumen was washed with ice cold HBSS to remove colon contents. Following removal of all visible contents, the serosa was separated from the mucosa using forceps and discarded. A surgical scalpel (no. 20) was used to segment the colon mucosa into 1 cm × 0.5 cm strips. Explants were then placed on a 4 cm × 2 cm block of agar (Fisher Scientific Ltd, Nepean, ON, 1% v/v in water) in a petri dish (60 mm × 15 mm, VWR International, Edmonton, AB) containing culture media (KBM Bullet Kit, Lonza, Walkersville, MD, USA), supplemented with 1.5 mM calcium. Media was further supplemented with one of the following combinations of antimicrobials: none, 50 μg/ml of gentamicin only, Mix 1 (200 μg/ml spectinomycin, 6.25 μg/ml vancomycin, 6.25 μg/ml colistin, 25 μg/ml spiramycin and 12.5 μg/ml rifampicin), and Mix 2 (consisting of Mix 1 supplemented with 10 μg/ml gentamicin and 10 μg/ml pimaricin). The model reported here is suggested to be used as an alternative model for swine associated spirochetosis, thus the antibiotic mix used in our experiments was previously reported to be selective for spirochaetes (Rubin et al., 2013a). The volume of culture media and block height were such that the explant was kept from being in direct contact with the media. Dishes were placed in a Modular Incubator Chamber (Billups-Rothenberg Inc., Del Mar, CA,

USA) and flushed with 95% O_2, 5% CO_2 mixture for 3 minutes (Figure 5.1). Incubation was carried out at 37°C. Gas mix and media were replaced every 24 hours. A summary of all samples collected is presented in Table 5.1. Explants were cultured from 12 hours to up to 9 days. Explants were harvested after each 24 hour period (except for day 6), fixed using 10% buffered formalin and submitted for routine paraffin waxing and H&E staining. From each pig, a segment of tissue processed but not cultured was processed as described and used as a control.

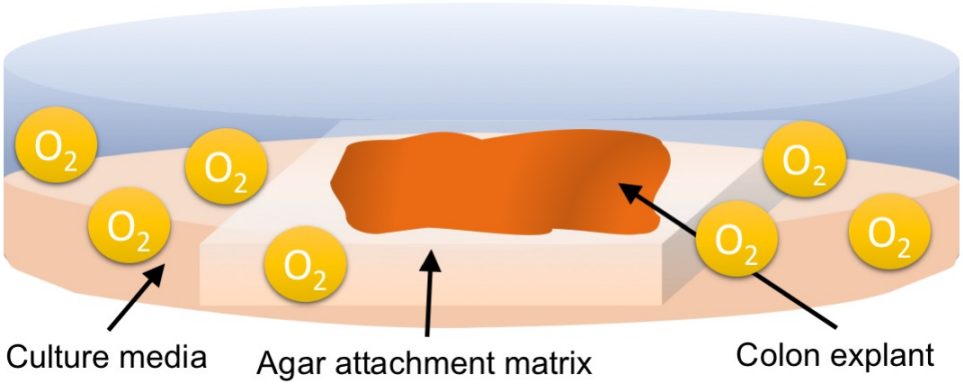

Figure 5.1. Explant culture setup.

Colon explants were placed on top of an agar matrix, isolating the tissue from direct contact with the media. An oxygen-rich gas (95% O_2, 5% CO_2) prevented drastic pH changes in the media due to secretin of metabolites by the tissue.

Table 5.1. Summary of samples collected for protocol development experiments (Experiment 1) and conditions applied to each.

Pig	Herd	Age at euthanasia (weeks)	Colon segment	Transport (hours)	Yielded explants (n)	Antibiotic[a]
1	1	7	Proximal	0	11	None
2	1	8	Distal	0	13	Mix 2
3	1	8	Distal	0	13	None
4	1	9	Distal	0	12	Mix 2
5	1	9	Distal	0	12	Gentamicin
6	1	10	Distal	0	13	Gentamicin
7	1	5	Distal	0	6	Mix 1
8	1	6	Distal	0	2	Mix 1
9	2	6	Apex	0	15	Mix 1
10	2	5	Proximal	0	2	Mix 2
				2	4	
			Apex	0	5	
				2	5	
				4	5	
			Distal	0	4	
				2	3	
				4	7	
11	2	6	Proximal	0	4	Mix 2
				2	4	
				4	4	
			Apex	0	4	
				2	4	
				4	4	
			Distal	0	4	
				2	4	
				4	4	

[a] Gentamicin - 50 µg/ml of gentamicin only. Mix 1 - is 200 µg/ml spectinomycin, 6.25 µg/ml vancomycin, 6.25 µg/ml colistin, 25 µg/ml spiramycin and 12.5 µg/ml rifampicin. Mix 2 - Mix 1 supplemented with 10 µg/ml gentamicin and 10 µg/ml pimaricin.

5.3.2.4 Evaluation of explant epithelium coverage

An examiner blinded to slide identity evaluated the H&E stained explant sections. The examiner acquired high-resolution electronic images for all samples, covering the entire explant mucosal surface. ImageJ software was used to measure the amount of surface covered by columnar epithelium. Values obtained were used to calculate the percentage of explant mucosal surface covered by epithelial cells, the surface epithelium coverage score (Girish and Vijayalakshmi, 2004). Analysis was carried out by grouping explants into two categories: ≥ 50% of the surface covered by epithelium or < 50% of the surface covered by epithelium.

5.3.3 Experiment 2 - Extended incubation trial

5.3.3.1 Pigs and animal care

Thirteen five-week old, crossbred male piglets were purchased from Herd 1. Animals were housed at the Animal Care Unit and acclimatized for 5 days. Pigs were monitored daily for health status. They were fed commercially prepared, non-medicated, pelleted starter diet *ad libitum,* and housed in 5′×8′ pens, each containing 4 pigs. One pig (#10) presented with lameness during the study. Treatment was attempted but no improvement was seen following treatment, so the pig was euthanized for humane reasons. Despite the antibiotic treatment, data from this pig was used in the study.

5.3.3.2 Colon explant culture

Pigs were euthanized by exsanguination after concussion by captive-bolt. Immediately after euthanasia one 10 cm segment of the distal spiral colon was harvested each pig, placed in ice-cold HBSS buffer supplemented with 1.5 mM calcium and transported immediately to the laboratory. At this point, a sample of colon was fixed in 10% buffered formalin to be used as a control, since it was exposed to minimal manipulation. Each colon segment was opened along the mesenteric border and luminal contents were washed by agitation in 200 mL of cold HBSS buffer. The serosa was separated from the mucosa, and the latter was sectioned into sixteen 0.5 cm × 0.5 cm explants. Explants were then placed in culture media (KBM Bullet kit) supplemented with 1.5 mM calcium and antibiotic Mix 2. Incubation was performed at 37°C in a 95% O_2 / 5% CO_2 atmosphere for up to 5 days. During this period, media and gas mix were replaced every 24 hours. A total of 208 explants were cultured from the thirteen pigs.

At day 0, two explants per pig were fixed in 10% buffered formalin, and four were fixed in RNAlater (Qiagen Inc., Toronto, ON). At day 5, four explants per pig were fixed in 10% buffered formalin, and six explants were fixed in RNAlater.

5.3.3.3 Histology and Ki-67 immunohistochemistry

All histological analysis was performed by an examiner blinded to sample identification. Epithelium coverage was evaluated based on the percentage of the explant section surface covered with columnar epithelial cells as described for Experiment 1. Immunohistochemistry was performed to detect cell proliferation marker Ki-67, and immunostained sections were

examined by optical microscopy. Five crypts per explant section were analyzed for the presence of Ki-67 staining in their nuclei as follows. Crypts were virtually divided on their sagittal planes, dividing crypts into halves, and 5 cells from each side of the plane (starting at the base of the crypt and moving towards the top) were evaluated for staining, resulting in a score of 0-5. An average crypt score was calculated for each pig at each time point based on the average scores calculated from the 5 crypts of each explant.

5.3.3.4 Quantitative PCR

RNA was extracted from samples preserved in RNAlater using a commercial kit (RNeasy, Qiagen Inc., Toronto, ON) and quantified by spectroscopy (Nanodrop 2000, Fisher Scientific, Wilmington, USA). An aliquot containing 20 mg of RNA from each sample was used to generate cDNA (iScript, Bio-Rad Laboratories Ltd., Mississauga, ON). Quantitative PCR assays targeting porcine GAPDH, E-cadherin, IL-8, IFNγ, TNFα and IL-1β are described in Table 5.2. The efficiency of all assays was determined using ten-fold dilution series of plasmids containing the target sequences; all were between 95% and 103%. Quantitative PCRs were conducted on a Bio-Rad CFX instrument with iQ SYBR green supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario). Each reaction consisted of 12 µL of 10× IQ SYBR Green Supermix, 2 µL of forward primer, 2 µL of reverse primer, 1 µL of cDNA template and 8 µL of water. Reactions were incubated at 94°C for 3 min, followed by 40 cycles of [30 sec at 95°C, 30 sec at 64°C and 30 sec at 72°C] and a final extension for 5 min at 72°C. All reactions were run in duplicate, and both extraction negatives and no template controls were included for each assay. Reaction duplicates that differed by more than 1 Cq value were repeated. Analysis of gene expression data

was performed using the ΔΔCq relative expression method with GAPDH as the reference gene (Livak and Schmittgen, 2001).

Table 5.2. Primer sequences used for gene expression analysis.

Target	Primer sequence (5'-3')	Amplicon size (bp)	Reference
IL-8	ATA CGC ATT CCA CAC CTT TCC ACC	159	Enns, Unpublished
	TCT GTA CAA CCT TCT GCA CCC ACT		
IFN-γ	ATG GTA GCT CTG GGA AAC TG	171	Enns, Unpublished
	TCT GGC CTT GGA ACA TAG TC		
TNF-α	ACG CTC TTC TGC CTA CTG CAC TTC	162	Enns, Unpublished
	TCC CTC GGC TTT GAC ATT GGC TAC		
GAPDH	ACA TCA AGA AGG TGG TGA AGC AGG	162	This study
	TGA GCT TGA CGA AGT GGT CGT TGA		
IL-1-α	GTG CTC AAA ACG AAG ACG AAC C	110	Duvigneau, 2005
	CAT ATT GCC ATG CTT TTC CCA GAA		
e-cadherin 1	ACA GGC ACC CTT CTC CTG TT	207	This study
	TTC TCG GTC GTT GAA CTC GAT GG		

5.3.3.5 Statistical analysis

Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL). H&E epithelium coverage between day 0 and day 5 and Ki-67 scores between day 0 and day 5 were compared using Kruskal-Wallis and Mann-Whitney post-hoc tests. *P* values less than 0.05 were considered significant.

5.4 Results

5.4.1 Experiment 1 – Exploring factors affecting explant survival

Overgrowth of fungi was observed on all explants from two pigs (n=24/pig) within the first 48 hours of culture. Another nine explants from three different pigs (#3, #4 and #11) were heavily contaminated with fungi (n=7) or Gram-positive bacteria (n=2) within the first 48 hours of culture. These contaminated tissues were not included in the study, leaving a total of 168 explants incubated for periods ranging from 0.5 to 9 days for analysis.

Explant viability was assessed by surface epithelial coverage at the end of the incubation period. Results are presented in Table 5.3. Overall, only 44% (74/168) of explants had ≥50% epithelial coverage, and pigs older than 7 weeks at euthanasia accounted for 70% (52/74) of these high quality explants (Figure 5.2). Interestingly, pigs from Herd 1 and 2 yielded 74% (61/82) and 15% (13/86) of explants with ≥50% of epithelial surface coverage, respectively (Figure 5.3). Pigs

from Herd 1 constituted most of the individuals older than 7 weeks of age. Thus, we were unable to separate the effect of pig age and farm upon tissue survivability. Explant viability degraded as time in culture increased (Figure 5.4). Nearly all (53/54, 98%) of the explants cultured for more than 5 days had <50% of their surface covered by epithelium. Despite the requirement for antibiotics to control microbial overgrowth, their addition to the culture media decreased the number of explants with high epithelial coverage (Figure 5.5). When no drugs were added, 75% (14/19) of the explants had ≥50% epithelial coverage. With gentamicin only added to the media, 90% (26/29) of the explants were in this category. When Mix 1 and Mix 2 were included in the culture media, only 50% (18/18) and 19% (16/84) had epithelium scores ≥50%, respectively. Proximal and apex segments yielded 27% (8/29) and 21% (9/42) high quality, while 59% (57/97) of distal colon explants had epithelium coverage of ≥50% (Figure 5.6). Processing explants immediately after euthanasia resulted in 56% (67/119) of explants with ≥50% epithelial coverage. Explants processed after 2 and 4 hours had 12% (3/24) and 16% (4/25) of explants with ≥50% scores (Figure 5.6).

Table 5.3. Explants epithelium scores under sample collection and culture conditions studied.

		Epithelium score		
Studied factor	**Treatments**	**< 50 % (n)**	**≥ 50 % (n)**	**Total**
Age (weeks)	5	34	9	43
	6	34	5	39
	7	5	8	13
	8	10	16	26
	9	11	23	34
	10	0	13	13
	Total	**94**	**74**	**168**
Age Group	≤ 7 weeks	73	22	97
	> 7 weeks	21	52	71
	Total	**94**	**74**	**168**
Culture period	0	0	4	4
	0.5	0	4	4
	1	10	30	40
	2	9	17	26
	3	5	7	12
	4	5	3	8
	5	11	8	19
	7	11	0	11
	8	2	0	2
	9	41	1	42
	Total	**94**	**74**	**168**
Colon Segment	Proximal	21	8	29
	Apex	33	9	42
	Distal	40	57	97
	Total	**94**	**74**	**168**
Antibiotic	None	5	14	19
	Gentamicin	3	26	29
	Mix 1	18	18	36
	Mix 2	68	16	84
	Total	**94**	**74**	**168**
Farm	Herd 1	21	61	82

	Herd 2	73	13	86
	Total	**94**	**74**	**168**
Time (hours)	0	52	67	119
	2	21	3	24
	4	21	4	25
	Total	**94**	**74**	**168**

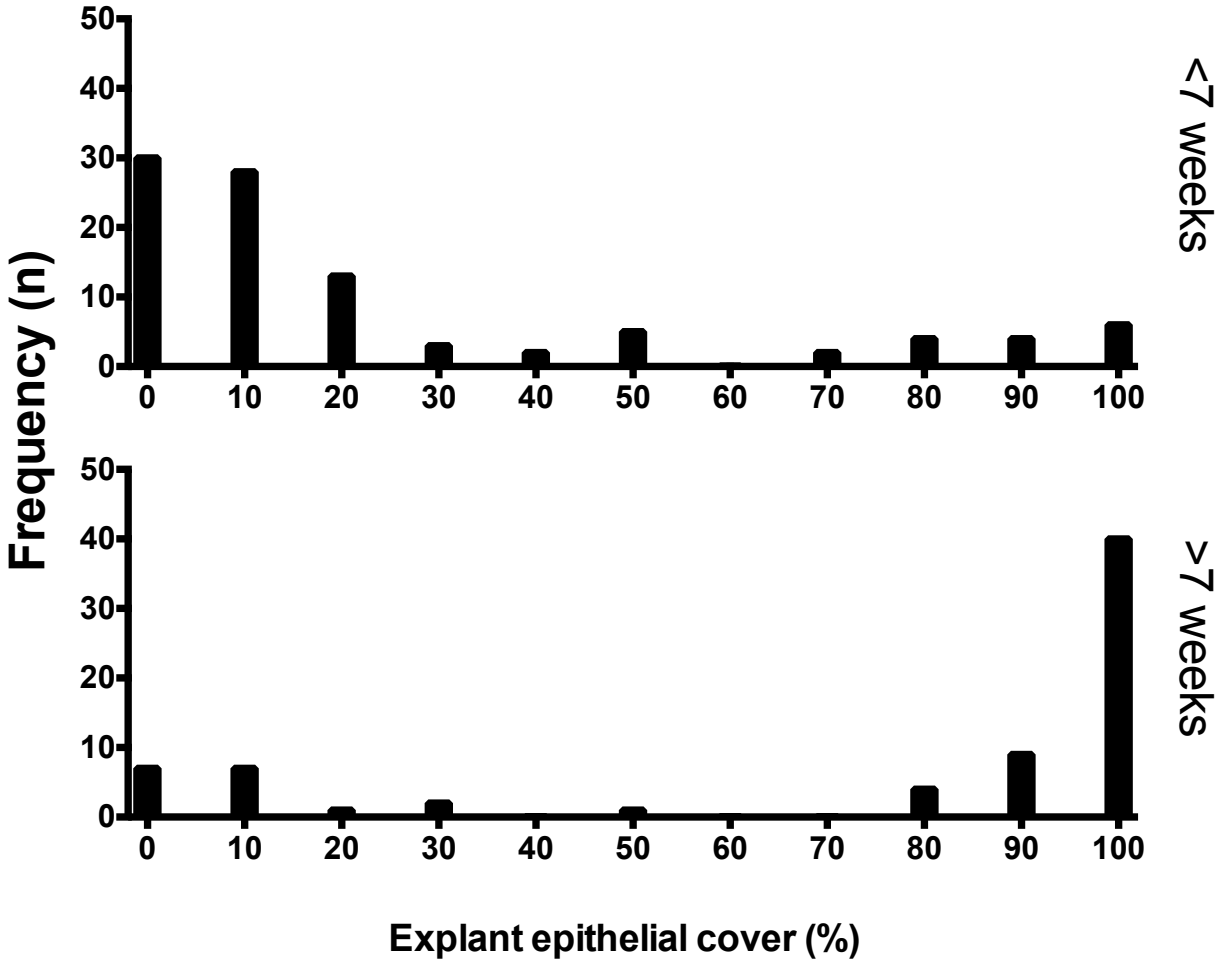

Figure 5.2. Frequency distribution of explant epithelial scores across pig age at euthanasia.

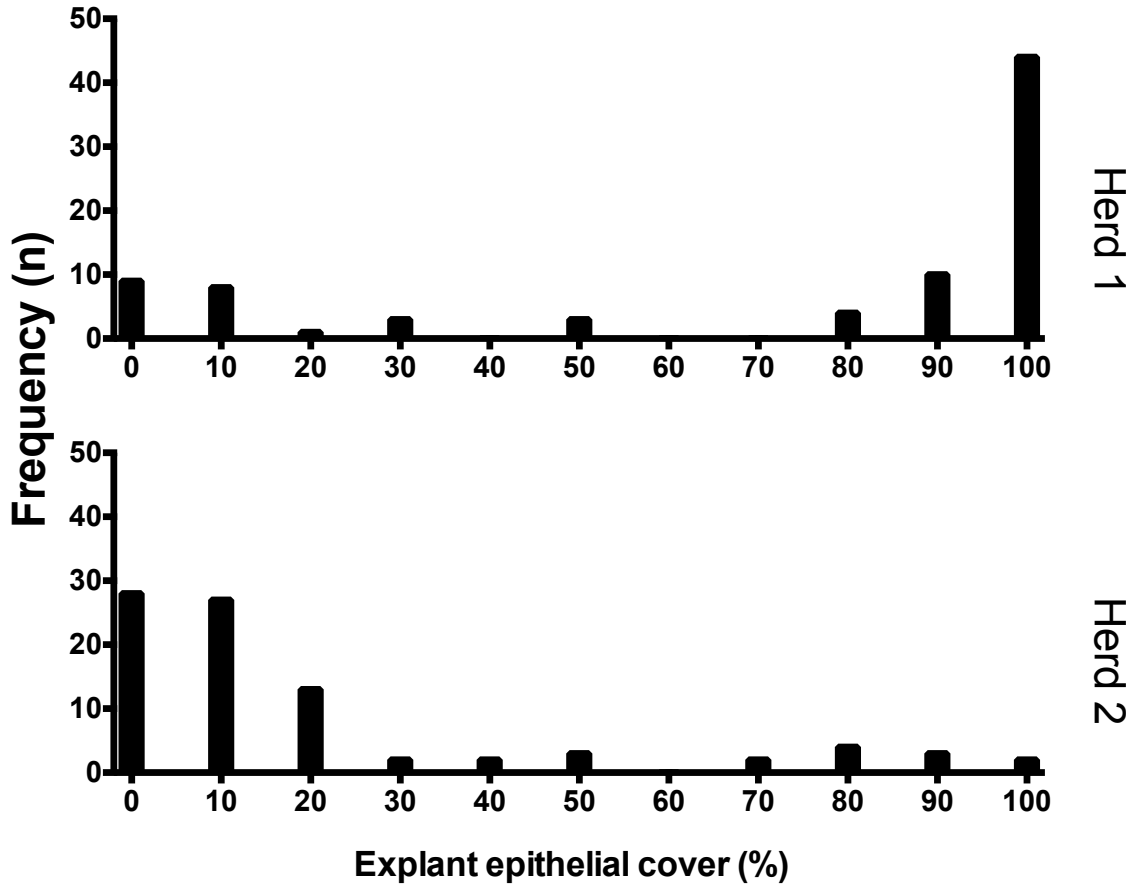

Figure 5.3. Frequency distribution of explant epithelial scores of explants prepared from pigs purchased from Herd 1 and Herd 2.

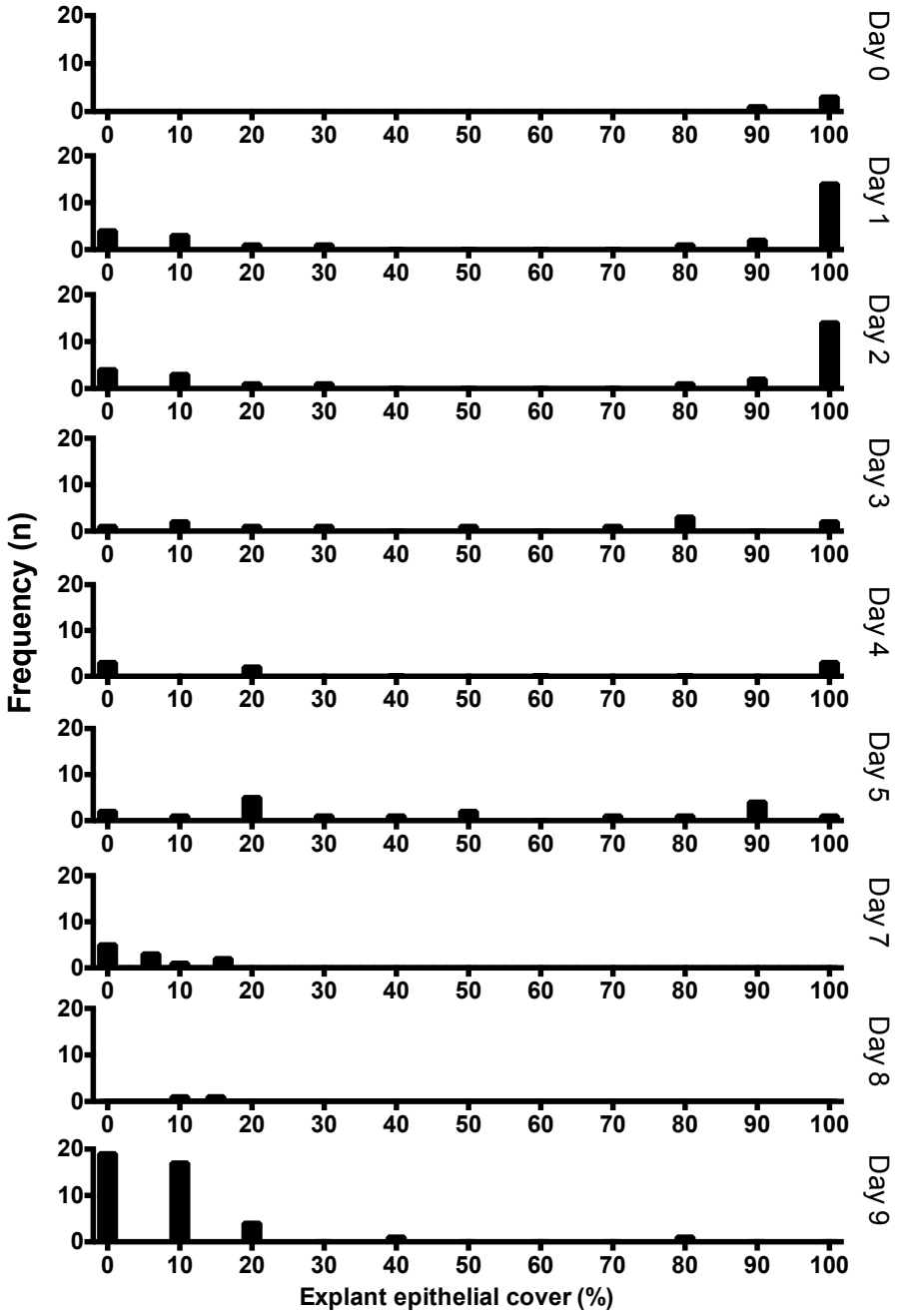

Figure 5.4. Frequency distribution of explant epithelial scores across explant time in culture.

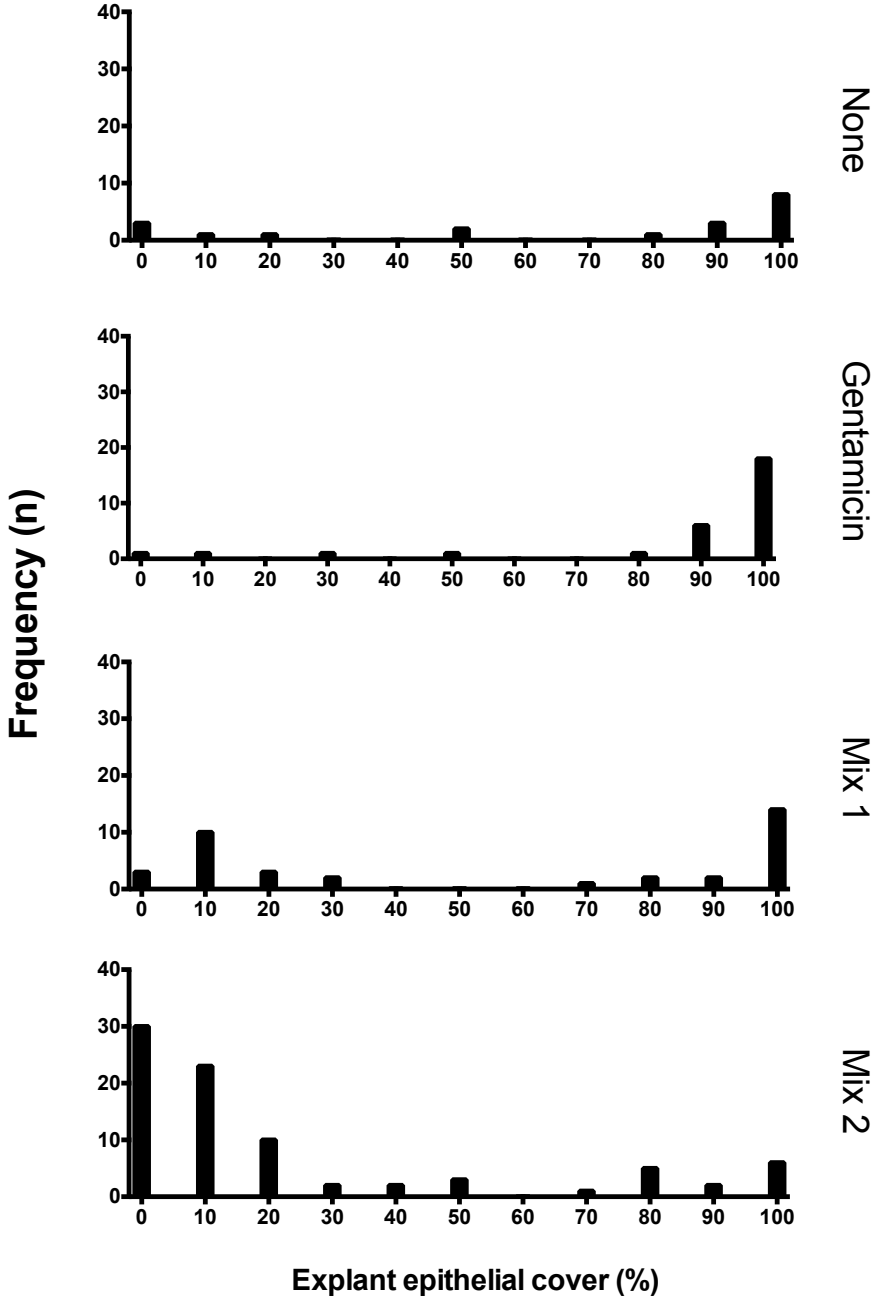

Figure 5.5. Frequency distribution of explant epithelial scores when different antibiotic drugs were incorporated in the culture media.

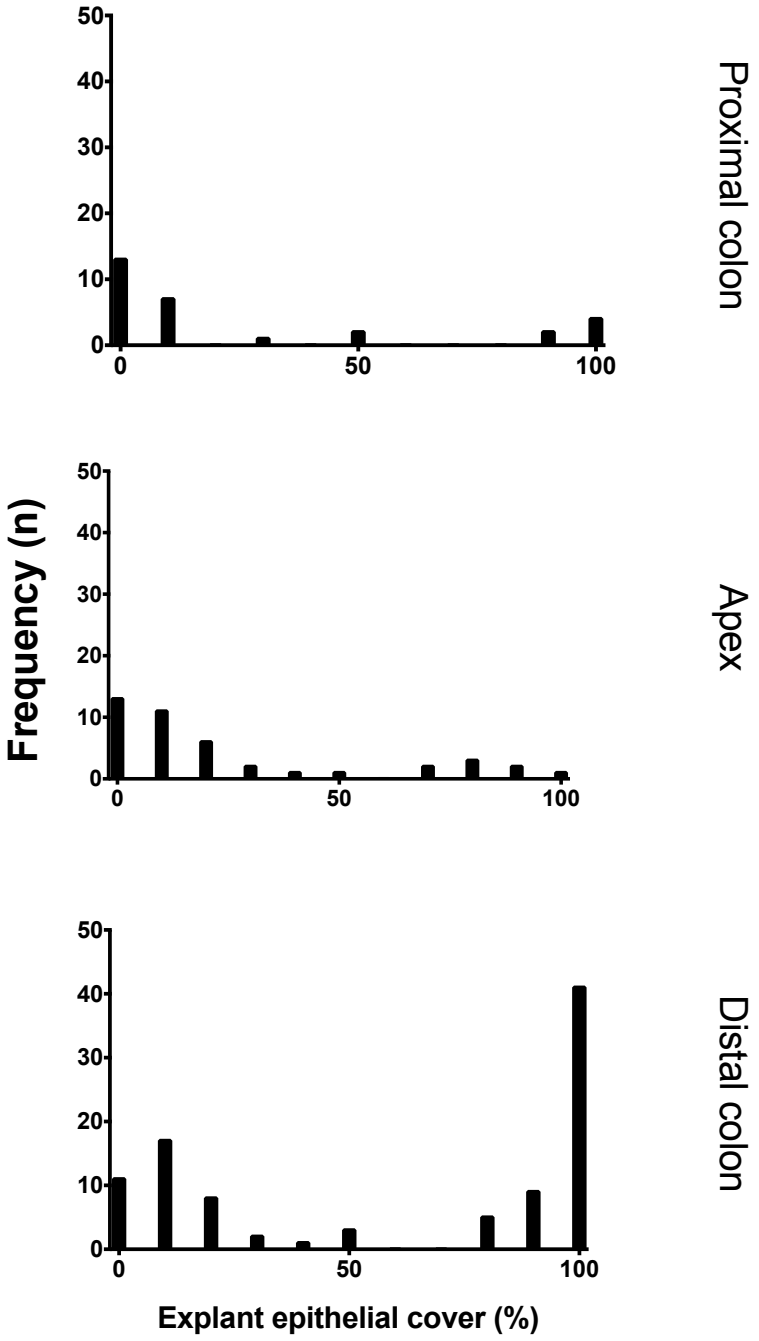

Figure 5.6. Frequency distribution of explant epithelial scores of explants obtained from proximal colon, apex and distal colon.

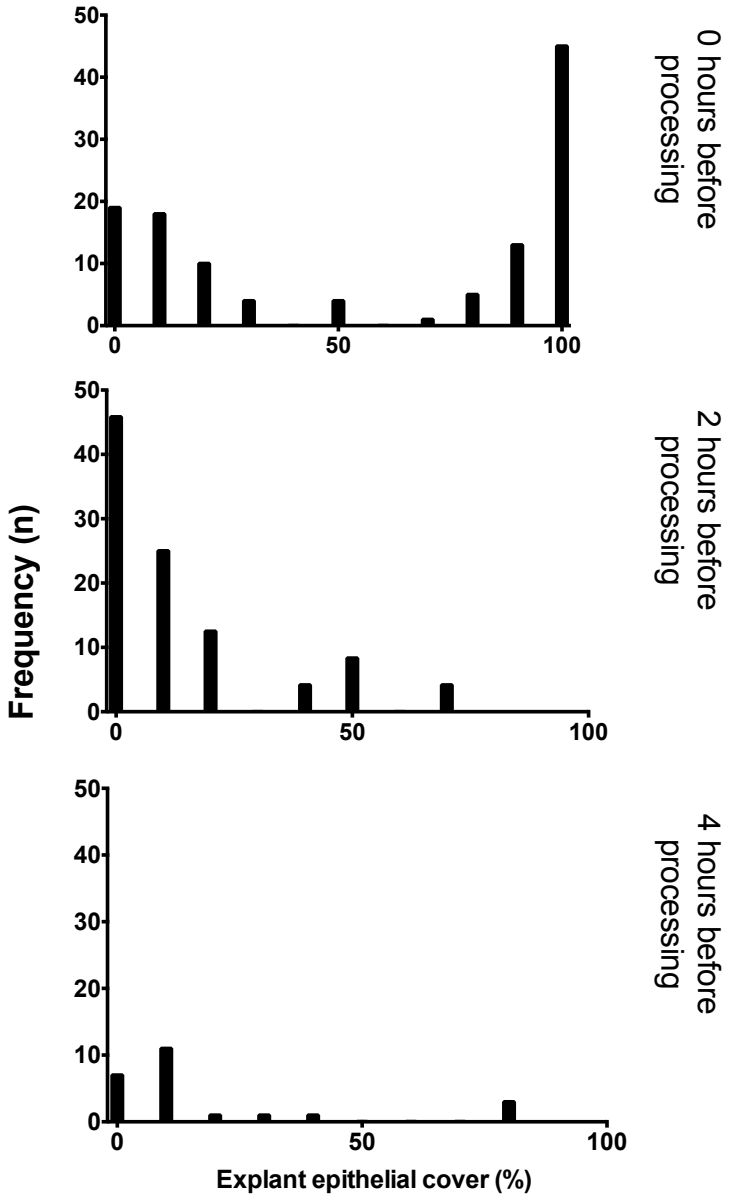

Figure 5.7. Frequency distribution of explant epithelial scores when segments were processed immediately after euthanasia (0 hours), after 2 and 4 hours.

Taken together, the results suggest that the greatest chance of culturing an explant with ≥50% epithelial coverage would occur using distal colon tissue from pigs euthanized after 7 weeks of age, processed immediately after euthanasia and kept in culture media supplemented with gentamicin for up to 24 hours. For longer culture periods of up to 5 days, addition of more antimicrobial drugs to the culture medium avoids bacterial overgrowth and death of explants, despite losses in explant epithelial cover.

5.4.2 Experiment 2 - Extended incubation trial

All pigs remained free of gastrointestinal clinical signs throughout the study period. Thirteen pigs were euthanized over 1 month: four pigs at 6 weeks of age, four at 7 weeks of age and five at 9 weeks of age. In total, 13 distal colon segments were successfully harvested and transported to the laboratory, providing enough tissue for 208 explants (16 explants per pig). Despite the use of antimicrobials in the media and aseptic tissue handling technique, culture contamination was observed occasionally. Pigs #1, #2 and #13 had one out of the initial 16 explants contaminated each, while pigs #10 and #11 had three and two explants contaminated, respectively. The most common contaminants observed were Gram-positive rods (n=5), followed by hyphal fungi (n=3). Upon observation of contamination (cloudy media, macroscopic colony formation on the explant or agar block and microscopic visualization by Gram staining) the explant was immediately removed from the incubator and discarded to avoid cross contamination of other explants.

Analysis of H&E stained sections revealed that day 0 samples were remarkably similar to healthy colon tissue fixed in formalin without any handling after euthanasia (Figure 5.8, day 0). Day 0 samples were processed and handled for processing identically to day 5 samples. A

summary of explant surface epithelial coverage per pig at day 5 is shown in Figure 5.9. Thirty-one percent (15/48) of the explants had ≥50% of their surface covered by columnar epithelium after 5 days in culture. Crypts were present in 81% of the explants at day 5. Histology analysis showed that metaplasic tissue, from columnar to cuboidal epithelium, was only observed in 4% of the explants (data not shown).

Expression of Ki-67 was analyzed as a marker for proliferative epithelial cells. The average number of Ki-67 positive cells at the crypt base was greater in day 0 samples than day 5 ($P<0.05$, Figure 5.10).

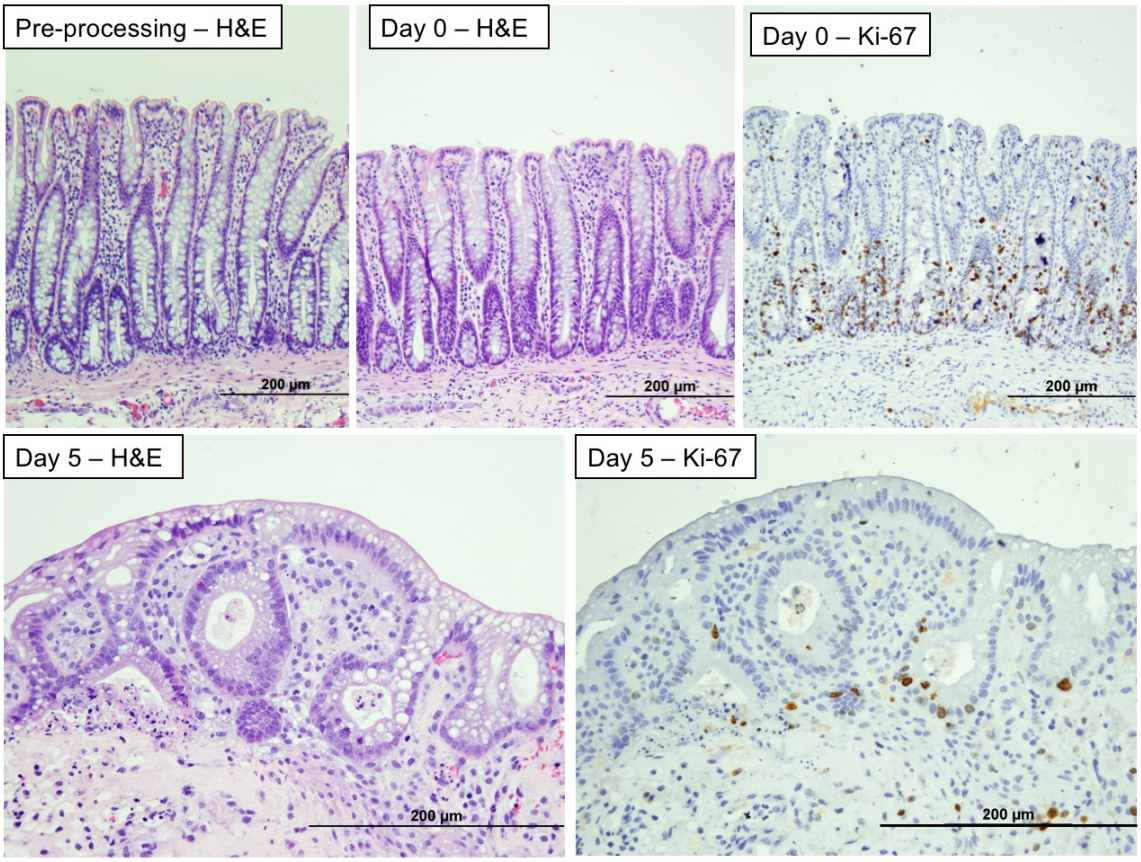

Figure 5.8. Explants histology.

H&E and Ki-67 stained sections of explants. Day 0 explants display the typical colonic pattern of crypts covered by epithelium, with the majority of Ki-67 cells present in the crypt base. Day 5 explants display crypts covered by epithelial cells and goblet cells. Ki 67 immunostained cells are present at the base of crypts and at the lamina propria.

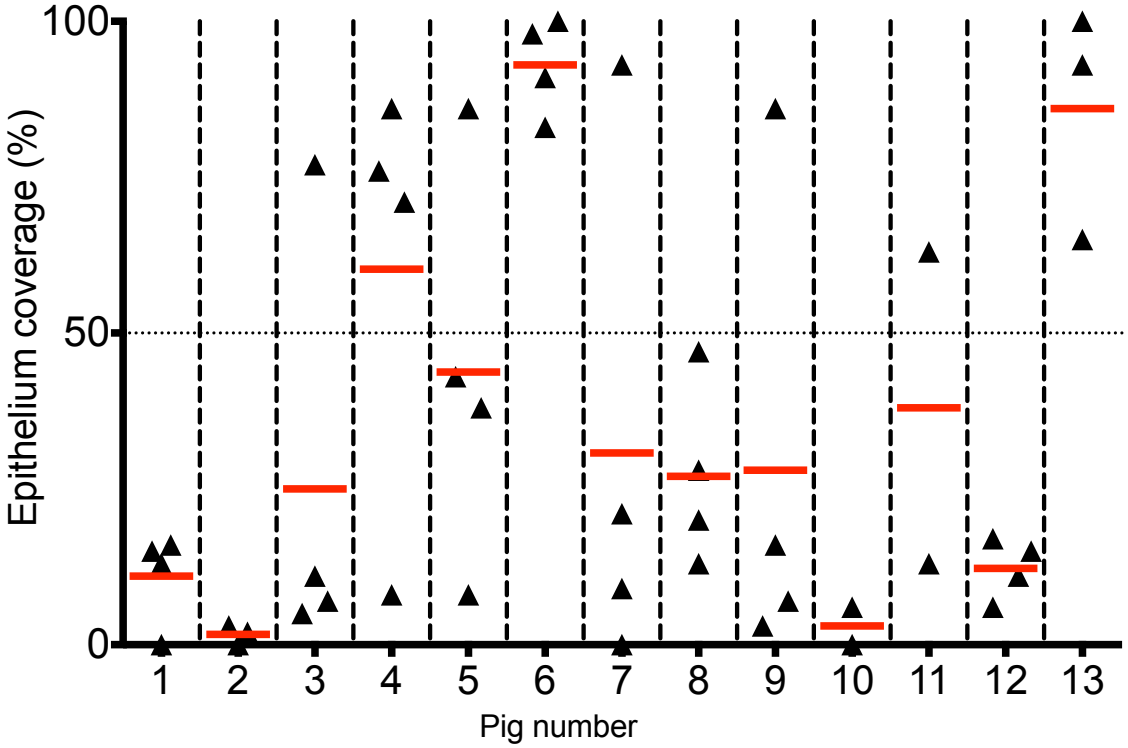

Figure 5.9. Percentage of explant surface covered by epithelial cells after 5 days in culture.

Each data points reflect an H&E section score obtained from one explant. Red bars denote the mean explant coverage for a given pig, at day 5.

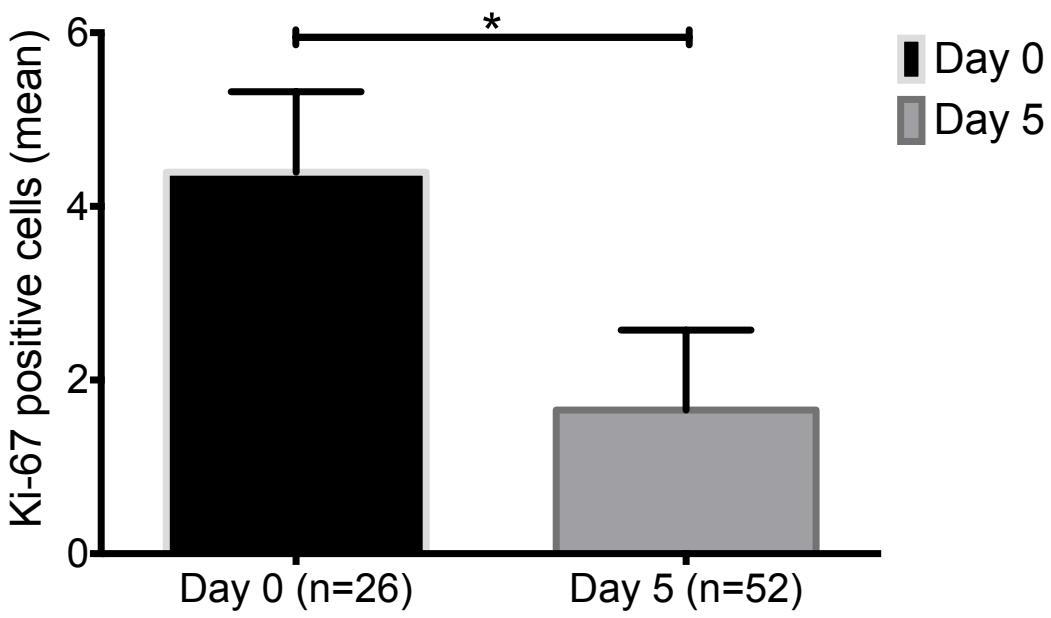

Figure 5.10. Mean number of Ki-67 positive cells at the crypt base.

Crypts from immunostained sections were evaluated for the number of cells at the base positive for the Ki-67 protein. A virtual sagittal cut divided the crypts (5 crypts per section) into two halves and the first 5 cells from the crypt base on each side were evaluated for Ki-67 staining. Scores range from 0 to 5. Bars represent mean values and standard deviations.

Explants had detectable levels of GAPDH mRNA on both day 0 and day 5 (Figure 5.11). The average GAPDH Cq for day 0 samples was 22.4 ± 1.4, while day 5 samples averaged 21.3 ± 1.0. Explants from 8/13 pigs displayed ≤1 Cq difference between day 0 and 5. Expression levels of several genes encoding pro-inflammatory cytokines and apical junction complex marker e-cadherin were analyzed relative to GAPDH, in order to evaluate the inflammatory response and epithelium integrity in cultured tissues. Duplicate Cq values obtained from all explants from each pig at a given time point were averaged and used for calculation of fold differences (Figure 5.12). Levels of e-cadherin and INFγ mRNA were marginally decreased between day 0 and day 5 (mean fold changes were 8.7 and 8.0, respectively). Mean TNFα expression remained within 2-fold difference between days 0 and 5 in culture, although a great deal of variance was observed among samples. IL-1 and IL-8 mRNA levels were higher on day 5 than day 0, with mean fold changes of 5.3 and 29.5, respectively.

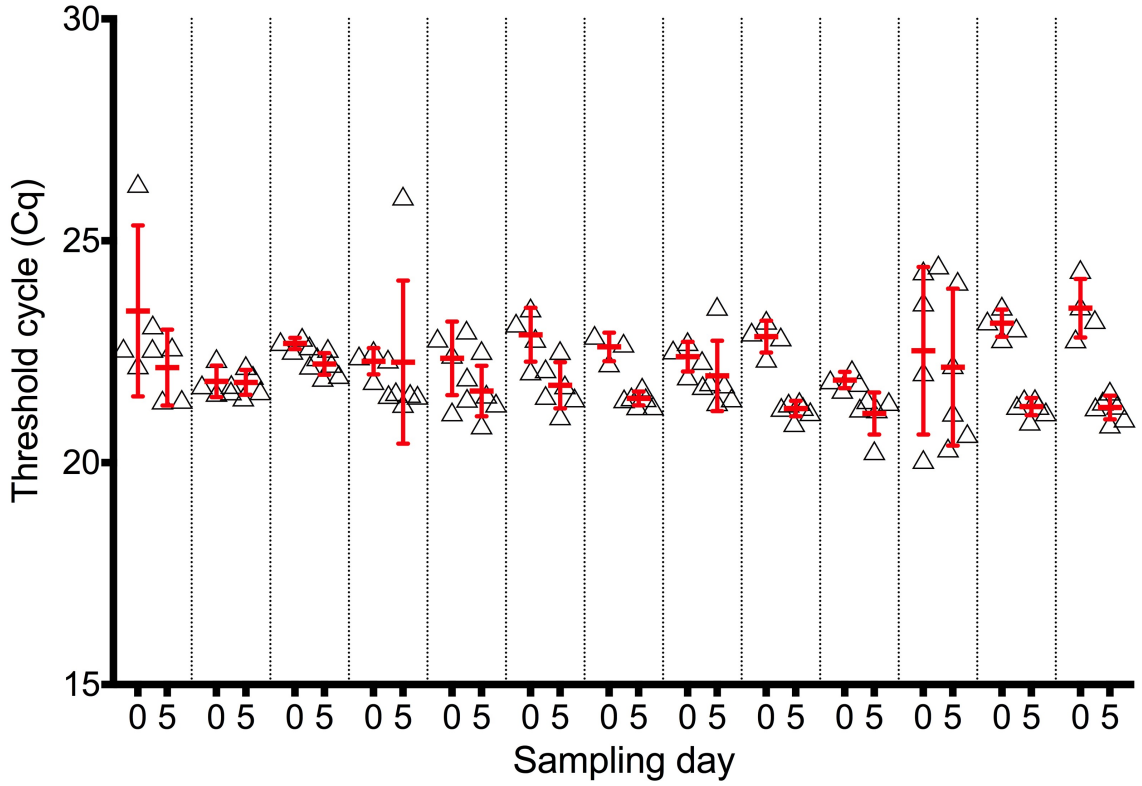

Figure 5.11. GAPDH gene expression by tissue explants at day 0 and day 5.

GAPDH gene expression was detected by quantitative PCR. Data points plotted are average Cq values from duplicate reactions for each explant, redlines denote mean and standard deviation for samples from a given animal at a given day. Higher threshold cycles indicate lower starting quantities of GAPDH mRNA.

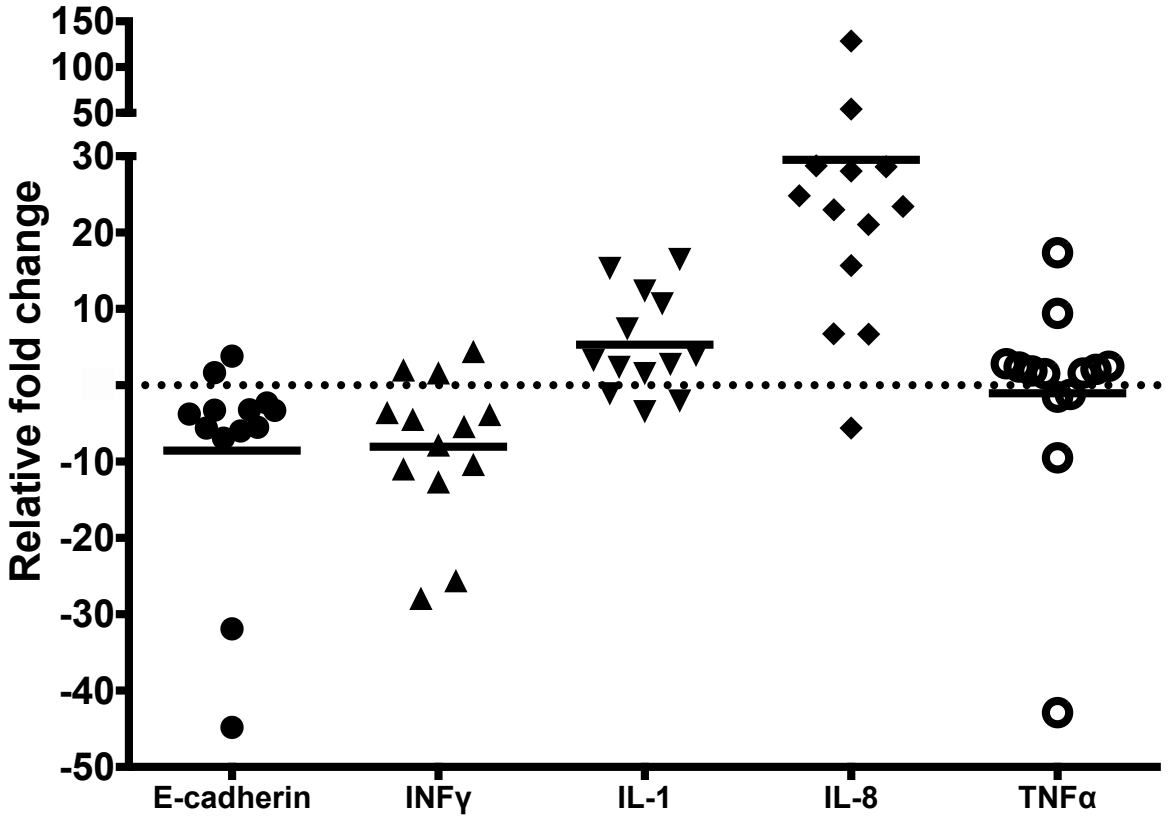

Figure 5.12. Relative gene expression fold changes.

Expression of different genes from explants sampled at day 0 and day 5 were compared relatively to the expression of GAPDH by the same explant at the respective sampling day. Data points are average values from all explants (n=10), from a pig for a given time point, analyzed using ΔΔCq method. Bars represent mean values for that gene in all pigs (n=13).

5.5 Discussion

Replacing and reducing animal use in research is a major concern of scientists worldwide, and *in vitro* cell culture models have been developed and applied to help achieve this objective. Despite the huge contributions brought by these models, they are deficient in the aspect that a single layer of cells is very different from a complex three-dimensional tissue (Abbott, 2003). *In vitro* organ cultures are not a new technique, as over a century ago Thomson described a protocol for culture of embryonic chick toes (Thomson, 1914). *In vitro* organ culture offers an attractive alternative to *in vivo* models, since its histologic characteristics are similar to *in vivo* tissue, but in a controlled environment. Even though animals are still required for tissue harvesting, the number of individuals can be greatly reduced using this modelling approach since one single donor may yield dozens to hundreds of explants, depending on available resources and animal size.

A number of other methods have been described previously for culturing adult human and rodent colonic tissue, as well as swine small intestine. In these reports, colon explants were kept alive *in vitro* for no longer than 60 minutes, with no visible disruption of the intestinal epithelial layer (Autrup et al., 1978a; Collins et al., 2010; Danielsen et al., 1982; Kik et al., 1991; Shamsuddin et al., 1978). In the current study, we evaluated different factors affecting the maintenance of porcine colon explants *ex vivo.* A heterogeneous population of tissue donor pigs was employed, which contributes to overall generalization of the outcomes observed. Finally, we proposed a protocol that can yield colonic mucosal explants with typical tissue architecture after 5 days *in vitro*.

As previously observed by Fell, there are many factors that influence tissue survivability *in vitro* (Fell, 1963). Previous studies suggest that organism size may play an important role in tissue survivability *in vitro*, e.g. human explants are less likely to survive than rodent explants (Autrup et al., 1978a; Autrup et al., 1978b; Fletcher et al., 2006). We observed that donor age had a significant influence on explant survival time. Foetal explants have been reported to maintain the full repertoire of cells for 2 weeks *in vitro* (Abud et al., 2005; Quinlan et al., 2006). The small size of embryo tissue and its physiological state of morphogenesis seems to contribute to tissue survival in the relatively anoxic *in vitro* environment. However, harvesting embryological tissue is technically and ethically more challenging than the use of adult biopsies or post-mortem tissue. Pigs used in this study ranged from 5 weeks of age (recently weaned) to 10 weeks of age (grower pig). At 5 weeks of age the colon undergoes notable microbiota and metabolic shifts due to a dietary change from milk to solid pelleted feed (Konstantinov et al., 2004; Pie et al., 2004). Pigs older than 6 weeks of age have likely recovered from the stress caused by dietary change, which may contribute to better explant survival. Although age seemed to be an important factor when sampling for explant culture, we also observed a relationship between age and herd. The majority of older pigs were obtained from Herd 1, and these rendered the highest epithelial scores. During this study, we were unable to differentiate which factor played the major role in explant survivability. Further studies are indicated to clarify the effect of donor age on explant culture success.

Results of previous studies suggest that the large intestine is more tolerant to *ex vivo* conditions than the small intestine. After 48 hours in culture, small intestine explants begin to deteriorate morphologically and to have reduced expression of enzymes (Mitchell et al., 1974; Zachrisson et

al., 2001), whereas Autrup and collaborators were able to maintain human colonic mucosa with relatively preserved morphology for up to 14 days (Autrup et al., 1978a). Other authors have modified Autrup's protocol and reported colonic rodent explants with normal morphology after 91 days *in vitro* (Shamsuddin et al., 1978). There is significant variability among studies in the length of time that explants remain viable in culture. Different studies have reported explants that have remained viable with relatively normal morphology for periods ranging from 14 to 91 days. Unfortunately, no data was shown regarding intra-study variability of explant survival (Autrup et al., 1978b; Reiss and Williams, 1979; Shamsuddin et al., 1978). The same studies reported progressive metaplasia in colon explants, where columnar epithelium is replaced by cuboidal epithelium, and crypts had reduced length or were absent. In this study, we observed metaplasia in only a small percentage (4%) of explants. However, the duration of incubation in this study was shorter than in studies that reported more significant metaplasia, so it is possible that our explants were at the beginning phases of metaplasia.

Anoxia and the associated oxidative stress are suggested as major sources of the ischemic and necrotic lesions observed *in vitro* (Shamsuddin et al., 1978). Under the conditions detailed in the methods section, 33% of explants displayed good epithelial coverage after 5 days in culture. Reiss and Williams (1979) reported that colon explants of conventional rats and mice, kept for 35 days in culture, had a survival rate of 30% and 20%, respectively. The same authors used an oxygen rich gas mix, a cellulose attachment matrix (pore size 0.8 μm), media supplemented with dexamethasone and serum, and intermittent exposure to gas by using a rocking platform. They also reported crypts in 16% of the explants from rats, and 11% from mice after 35 days incubated (Reiss and Williams, 1979). Our study did not employ serum in the media and explants were

constantly exposed to the gas mixture, resulting in a similar survival rate (33%) and a higher proportion of explants with crypts (81%).

We also investigated the survivability of explants from different anatomical regions of the spiral colon and found that distal colon explants had higher epithelial cover scores than other regions. Site selection frequently reflects the area of interest of each researcher and the objectives of their studies (Collins et al., 2010; Reiss and Williams, 1979; Shamsuddin et al., 1978). We have shown that culture of proximal segments of spiral colon and apex was feasible. However, we suggest that a greater number of explants should be prepared due to the relatively poor viability of proximal colon and apex explants.

Tissue transport time was found to be an important factor associated with explant survival. Our data showed that tissue kept in refrigerated media for 2 and 4 hours resulted in explants with lower scores than tissue processed immediately after euthanasia. The literature lacks other data regarding these pre-processing effects on tissue survival, but authors have suggested that the shorter the interval between harvesting and culturing, the more successful culture should be (Resau et al., 1991). These same authors postulate that extracellular calcium influx leads to irreversible cellular injury, and thus transport media should be supplemented with calcium (Yu et al., 1990).

Previous reports suggested the use of CMRL 1066, RPMI 1640 or DMEM at a pH of 7.2-7.4 as basal media. Basal media supplements are unquestionably necessary for any tissue culture *ex vivo*. Reiss and Williams showed the importance of insulin and corticoid for mucosal architecture maintenance, as well as the degradation of morphological features when explants were exposed

to fetal bovine serum (Reiss and Williams, 1979). Use of antibiotics is suggested at least during the initial periods of culture, including antifungal drugs. Episodes of contamination of all explants from a pig motivated us to include piramicin in the culture media, in order to control fungal growth. However, we observed lower epithelium coverage scores with the addition of drugs to the culture media. When Mix 1 or Mix 2 was added, 50% and 19% of the explants had greater than 50% of their surface covered by epithelium, compared to 75% and 90% when no drugs or gentamicin alone were included. The detrimental effect of antibiotics on the mucosa has been previously described *in vivo* (Wlodarska et al., 2011). Explants lack clearance mechanisms, such as blood circulation and excretion in the urine and/or bile, which probably enhances the cytotoxic effects of the drugs (Dobbins et al., 1968; Levy, 2000). As an alternative to supplementing culture media with antimicrobial drugs, the treatment of tissue donor animals with antibiotics prior to euthanasia has been suggested (Reiss and Williams, 1979). Frequently reported antibiotic supplements for culture media include penicillin, streptomycin, amphotericin and fungizone. Regardless of the drug selection, authors have found it imperative to use antibiotics to avoid overgrowth of bacteria or fungi and the subsequent failure of the culture system (Abud et al., 2005; Appleton et al., 1991; Autrup et al., 1978a; Collins et al., 2010; Kik et al., 1991; Quinlan et al., 2006; Schiff, 1975).

Previous investigators suggested the use of semi-solid matrix to isolate the explant from the media, creating an air-liquid interface and improving oxygenation of the tissues. Different materials, such as collagen-coated plates, membrane inserts, gelfoam, fibrin foam and stainless steel grids have been used (Autrup et al., 1978a; Browning and Trier, 1969; Fletcher et al., 2006; Schiff, 1975; Shamsuddin et al., 1978). Nutrients and water are expected to reach explants

through the attachments matrix by diffusion. A previous study suggested that matrices with pore size of 800 nm resulted in explants with the highest number of viable crypts after 4 days in culture (Reiss and Williams, 1979). The agar block employed in this study is expected to have an average pore size of 350 nm, which is half the size reported for matrices previously used (Pernodet et al., 1997). Testing of 0.5% agar blocks, with greater pore size, may improve explant survival in future studies.

Expression of inflammatory and epithelial maturation markers was characterized by quantitative PCR data analyzed using the ΔΔCq method (Livak and Schmittgen, 2001). During the culture period explants are subject to oxidative stress due to the lack of blood oxygen supply, which contributes to cell death. When exposed to acute oxidative stress, IPEC-J2 cells respond by upregulating the expression of IL-8 and TNF-α (Paszti-Gere et al., 2012). Similarly, we observed increased expression of IL-8 and IL-1α over time. Eckmann et al. reported highly variable IL-8 expression detected by ELISA (ranging from 4.7 ng/mL to 134.6 ng/mL) from the supernatant of primary human intestinal epithelial cells after 48 hours in culture (Eckmann et al., 1993). We also observed great variance in the detection of IL-8. Different preparations from different donors yield heterogeneous populations of cells, especially given the distribution of mucosa associated lymphoid tissue in the colon. The lymphoid tissue, while being an important feature modeled by IVOCs, contributes to increased variance in the expression of cytokines given the resulting uneven distribution of immunoregulatory cells among explants. A very precise colon donor region for the preparation of explants should be delimited, in order to avoid such variation and thus establish a more controlled environment.

Ki-67 is a nuclear protein associated with cellular proliferation and ribosomal RNA transcription (Bullwinkel et al., 2006). In this study, immunohistochemistry was used to show that proliferative epithelial cells were present in the base of crypts of explants after 5 days of culture. This cell proliferation marker is key to demonstrate that explants were not only maintaining their cell population, but also building new cells over time. These cells are responsible for re-populating the surface of the explants with epithelial cells, as they migrate from the bottom of the crypts to the surface replacing sloughed off older cells. Other authors have reported the presence of Ki-67 positive cells in organ cultures of human skin and colon after 24 and 48 hours, respectively (Dame et al., 2011; Onuma et al., 2001). Ki-67 provides a useful reliable marker for quality check of culture protocols, as it reflects the tissue ability to replenish its cell population.

5.6 Conclusions

We have developed and demonstrated a protocol for successful culture of explants of porcine colon for up to 5 days. Additionally, we identified several factors that have an impact on explant survivability over time that should be considered when designing an organ culture system. Further improvements to the protocol described here are strongly encouraged, as the authors believe that a longer culture period with viable and differentiated epithelium will benefit future research.

5.7 Transition statement

Development of an *in vitro* model that has the similar characteristics to the infection site *in vivo* provides a powerful tool for further investigation of the host-pathogen relationship. Given the maintenance of the different cells and tissue architecture, it simulates the colonic lumen environment and thus stimulates pathogens to perform similarly as they would *in vivo*. We have described a technique that was able to support differentiated tissue for up to 5 days *in vitro*. In order to investigate the host mucosal response to the early events of "*B. hampsonii*" infection, a study was designed using the above-described model infected with pure cultures of the spirochaete.

6 Infection of porcine colon explants with *"B. hampsonii"* leads to increased epithelial necrosis and catarrhal exudate

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Conceived and designed experiments: MOC, JEH and JCSH. Performed experiments: MOC. Analyzed samples: MOC, RN and CF. Analyzed data: MOC, JCSH, CF. Wrote the paper: MOC, JEH.

6.1 Abstract

Mucohaemorrhagic diarrhea in pigs has a global distribution, and a remarkable economic impact on affected farms due to poor performance of animals. A critical knowledge gap exists regarding the pathogenesis mechanisms employed by *Brachyspira*. Multiple demonstrations that *"B. hampsonii"* strains are pathogenic to pigs have been achieved using *in vivo* animal models. In this study, we used *in vitro* organ culture of porcine colon to investigate the interactions between *"B. hampsonii"* strain 30446 and colon explants during the first 12 hours of contact. A total of 400 explants from ten 8-week-old pigs were prepared and randomly divided into two groups, inoculated and control. Inoculated explants received a 48-hour-old pure culture of *"B. hampsonii",* while controls received sterile broth. Explants were fixed in formalin (n=2/pig/time) or RNAlater (n=2/pig/time) after 0, 2, 4, 8 and 12 hours of incubation. Samples were analysed by optical microscopy (H&E and Warthin-Faulkner stains) and quantitative PCR (GAPDH, e-cadherin, IL-1α, IL-8, INF-γ and TNF-α mRNA). Significantly greater numbers of necrotic crypt cells and thicker catarrhal exudate were observed on infected explants compared to controls. Spirochaetes were observed in the mucus layer, in contact with necrotic exfoliated cells, in crypts and the lamina propria. No statistically significant differences were observed in mRNA levels between inoculated and control explants for any of the targeted genes. These results provide the first demonstration of a porcine colon explant model for investigating interactions of *Brachyspira* with its porcine host.

6.2 Introduction

Swine dysentery (SD) is an enteric disease of pigs that can cause great economic losses to pork producers through reduced feed conversion efficiency and increased production costs due to the use of antibiotics (Wood and Lysons, 1988). The syndrome was first described in the USA in 1921 but it was not until 1971 that two different groups simultaneously suggested *Treponema hyodysenteriae* (now *Brachyspira hyodysenteriae*) as the causal agent of SD (Glock, 1971; Taylor and Alexander, 1971; Whiting et al., 1921a). The classical description of the disease is mucohaemorrhagic diarrhea and colitis in grower and finisher pigs.

SD was a major concern among commercial pork producers in North America until the mid-1990s. The use of preventive antibiotic therapy associated with increased biosecurity likely contributed to a state of relative control of clinical SD in commercial farms. Feed and water supplemented with antimicrobials helped control pathogen populations to an extent that severe bloody diarrhea was not observed. However, since the mid 2000s swine veterinarians and producers have reported the re-emergence of mucohaemorrhagic diarrhea in pigs in North America. Initially, an increased number of mucohaemorrhagic diarrhea samples had detectable levels of *Brachyspira hyodysenteriae* and/or "atypical" *Brachyspira* spp. reported (Harding et al., 2010a; Harding et al., 2010b; Schwartz, 2011). Further characterization of these "atypical" *Brachyspira* isolates led to the proposal of the novel species "*Brachyspira hampsonii"* (Chander et al., 2012).

Since its initial identification, "*B. hampsonii"* has been demonstrated to be pathogenic to pigs, causing mucohaemorrhagic diarrhea indistinguishable from SD (Burrough et al., 2012a;

Burrough et al., 2012b; Costa et al., 2014; Rubin et al., 2013a). Despite multiple authors having replicated the disease using *in vivo* animal models, there remains a critical knowledge gap regarding the pathogenesis mechanisms employed by *Brachyspira* in causing mucohaemorrhagic diarrhea. Animal models have been very effective for demonstrating the consequences of *Brachyspira* colonization and infection of the swine colon, which are reported to occur over a period of 4-14 days after inoculation. However, this work has necessarily focused on the clinical disease period, and early host-pathogen interactions remain uncharacterized. *In vitro* models offer an alternative approach to addressing questions about early host-pathogen interaction. In particular, *in vitro* organ culture (IVOC) techniques enable whole organ explants to be maintained *ex vivo* for an extended period of time while preserving histological tissue features, offering a potentially powerful model system for studying the host-pathogen interaction (Trowell, 1959).

The objective of the current study was to use a porcine colon IVOC model to study the first 12 hours of interaction between "*Brachyspira hampsonii"* clade II strain 30446 and its porcine host.

6.3 Materials and methods

6.3.1 Animal care

Experiments were designed and conducted in accordance with the Canadian Council for Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply (Protocol 20130034).

Ten six-week old, crossbred male piglets were purchased from a PRRS negative, high health, commercial farm in Saskatchewan, Canada, with no history of spirochaete-associated diarrhea. Animals were housed at the Animal Care Unit, University of Saskatchewan, and acclimatized for 10 days prior to initiation of the trial. Pigs were monitored daily for gastrointestinal clinical signs and other signs of illness. They were fed commercially prepared, non-medicated, pelleted starter diet ad libitum, while housed in 4′×6′ pens, each containing 2 pigs.

6.3.2 Colon segment collection and explant preparation

Ten pigs were euthanized at 8 weeks of age, over a period of 7 days. Euthanasia was performed by exsanguination after captive-bolt desensitizing. Immediately after euthanasia, laparotomy was performed and a 15 cm segment of distal colon was excised. Tissue preparation for culture and explant culture were performed as previously described (Chapter 5). Briefly, the harvested distal colon segment was washed and the mucosa was surgically separated from the serosa. From each colon segment, explants (n=40) measuring 2 cm × 2 cm were prepared and placed on a 1% agar (v/v in water) block in a culture dish. Each dish received 6 mL of KBM Bullet Kit media (Lonza, Walkersville, MD) supplemented with 1.5 mM calcium ($CaCl_2$, Fisher Scientific Ltd, Nepean, ON) and an antibiotic mix selective for *Brachyspira* spp. (200 µg/ml spectinomycin, 6.25 µg/ml vancomycin, 6.25 µg/ml colistin, 25 µg/ml spiramycin, 12.5 µg/ml rifampicin, and 10 µg/ml pimaricin). Before incubation, a polystyrene ring (1 cm of diameter by 1 cm of height) was attached to the mucosal side of each explant using a cyanoacrylate adhesive (3M Vetbond Tissue Adhesive, 3M Animal Care products, St. Paul, MN). The adhesive was applied on the outer aspect of the ring, leaving the ring inner side adhesive-free while fixing the ring to the explant

(Figure 6.1). This apparatus prevented the inoculum from reaching the basolateral side of explants. Incubation was carried out in modular anaerobic chambers (Billups-Rothenberg Inc., Del Mar CA) infused with 95% O_2, 5% CO_2 at 37°C.

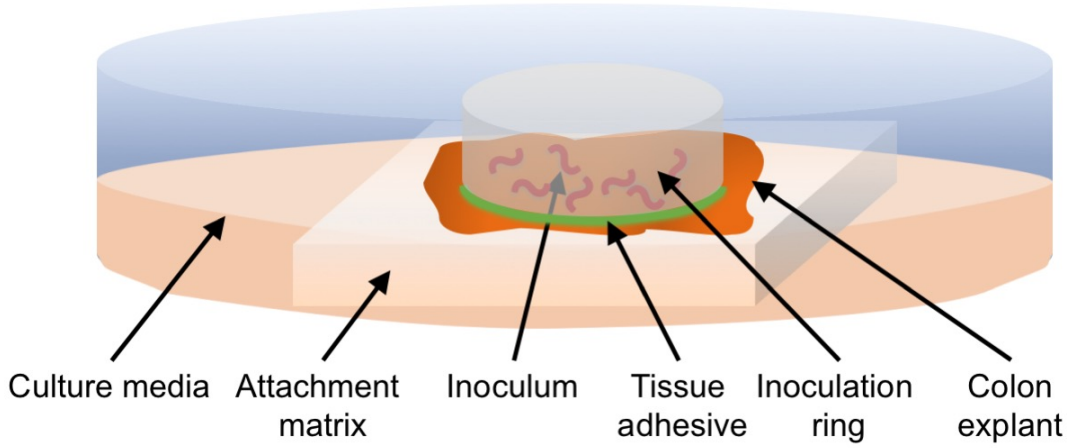

Figure 6.1. Explant inoculation setup.

6.3.3 Inocula preparation and explant infection

Pure cultures of "*Brachyspira hampsonii*" clade II strain 30446, an isolate proven pathogenic to pigs, were prepared as previously described (Rubin et al., 2013a). Briefly, spirochaetes were cultivated in glass vials containing 25 mL of brain heart infusion broth with 5% (v/v) fetal calf serum, 5% (v/v) sheep's blood, and 1% (w/v) glucose. Broth culture vials contained magnetic stir bars and were incubated anaerobically at 39°C for 48 hours with constant stirring. Immediately before infecting explants, culture vials were retrieved from the incubator and an aliquot was collected for verification of bacterial activity and quantification by species specific PCR. Phase contrast microscopy was used to visually assess spirochaete motility as an indicator of viability. Cultures containing active, motile spirochaetes were centrifuged for 5 minutes at 2500 × g. The resulting supernatant was discarded and the pellet resuspended in sterile phosphate buffered saline (PBS, 0.1 M, pH 7.0) and vortexed to resuspend the bacteria. In parallel, sterile culture broth was prepared and assessed the same way for use as a control.

Explants from each distal colon segment were randomly assigned to two groups: Infected (n=20/pig) and Control (n=20/pig). Each explant received 500 µl of inoculum that was carefully pipetted into the inoculation ring. Inoculated explants were incubated for 2 hours. After this period the inocula were removed by pipetting, and explants were returned to the incubator after re-filling the chambers with hyperoxic gas mixture. Explants were maintained in culture for a total of 12 hours. Sampling occurred at 0, 2, 4, 8 and 12 hours after inoculation. At each time point, two explants from each group were fixed in 10% neutral buffered formalin, and two were fixed in RNAlater (Qiagen Inc., Toronto, ON).

Inoculum viability at the beginning and end of the incubation period was assessed by culture of samples collected from the apical side of explants using a sterile loop as previously described (Rubin et al., 2013a).

6.3.4 Analysis of H&E and Warthin-Faulker stained sections

Histological analysis of explant morphology and spirochaete localization within explants was carried out using optical microscopy. H&E stained sections were used to calculate necrotic scores and to measure thickness of catarrhal exudate. Necrosis scores were based on evaluation of 25 crypts from each explant for which the entire crypt length was visible (score 0 - no necrosis, score 1 - < 10% necrotic cells, score 2 - 11-50% necrotic cells, score 3 - >50% necrotic cells). An average crypt score was reported for each explant. Catarrhal exudate thickness on the apical aspect of each section was measured using a microscope equipped with an intra-ocular micrometer within the eyepiece (Olympus WHN10X-H/22). Thickness was measured at five different locations along the length of the explant (far left, left, centre, right, far right). An average exudate thickness was reported for each explant.

Entire explant sections stained with Warthin-Faulkner silver staining (WF) were investigated for the localization of spirochaetes. An average score was reported for each pig (score 0 - no spirochaetes observed, score 1 - spirochaetes on apical epithelial surface only, score 2 - small numbers of spirochaetes in multiple glands, score 3 - many spirochaetes within several glands and score 4 - many spirochaetes forming thick mats in numerous glands).

6.3.5 Quantitative PCR

Analysis of gene expression in explants targeted the following genes: GAPDH, e-cadherin, interleukin-1α (IL-1α), interleukin-8 (IL-8), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) using previously described assays (Chapter 5). Total RNA extraction, sample preparation, gene expression analysis were conducted as described in Chapter 5. Quantification of *"B. hampsonii"* in the inoculum was performed as previously described (Rubin et al., 2013b).

6.3.6 Statistical analysis

Statistical analysis was performed using STATA 12.0 (College Station, TX, USA). Gene expression analysis was based on Cq values for each gene at a given time point relative to the Cq for GAPDH (ΔCq). Exudate thickness, necrosis score, spirochaete localization and ΔCq values from qPCR were compared between all time points and 0 hours, between groups (Infected and Control) regardless of time, and within time points between groups by generalized estimating equations (GEE, exchangeable correlation matrix).

6.4 Results

Ten 8-week-old healthy pigs were euthanized and colon explants were prepared for *in vitro* culture. From each pig, 40 explants were evenly divided between two groups: Control and Infected. Viability of the inoculum pre-inoculation was confirmed by culture on selective agar resulting in strong β-haemolysis. Explants from pigs #1 and #2 received 2.6×10^8 genome equivalents of *"B. hampsonii"*, explants from pigs #3 and #4 received 1.7×10^8 genome equivalents, explants from pigs #5 and #6 received 1.85×10^9 genome equivalents, explants

from pigs #7 and #8 received 2.0×10^8 genome equivalents and explants from pigs #9 and #10 received 1.7×10^8 genome equivalents. Samples were collected after 0, 2, 4, 8 and 12 hours of incubation and were either fixed in formalin (n=2/time point) or in RNA preserving solution (n=2/time point). No bacterial or fungal overgrowth was observed on explants during the experiment. Species-specific qPCR was used to determine the inoculum concentration. All samples collected after 12 hours of incubation from the Infected group displayed strong β-haemolysis after 48 hours of incubation on *Brachyspira* selective agar plates. Control group inocula resulted in no growth before or 12 hours after inoculation.

Analysis of H&E stained sections focused on the number of necrotic cells within crypts and the accumulated catarrhal exudate on the apical aspect of explants. Analysis of crypt cells revealed an increasing number of necrotic cells over time regardless of group, with all time points differing from time 0 hour ($P<0.001$, GEE). A significant difference was also observed between groups, when all time points were taken in account ($P<0.001$, GEE). When groups were compared within hours, significant differences were seen at 2, 4 and 8 hours ($P\leq0.001$, GEE) and a trend was observed at 12 hours ($P=0.07$, GEE, Figure 6.2). Catarrhal exudate analysis revealed an increasing thickness of exudate over time, with significant differences observed between all time points and the 0 hour samples regardless of group, as well as between groups when all time points were considered together ($P<0.01$, GEE). When grouped by time point, exudate thickness was greater in infected explants than controls after 4 and 8 hours of incubation ($P<0.05$, GEE, Figure 6.3), and a trend was observed at 12 hours ($P=0.06$, GEE). Exudate was characterized by variable amounts of mucus, necrotic and degenerated epithelial cells and bacteria (Figure 6.4).

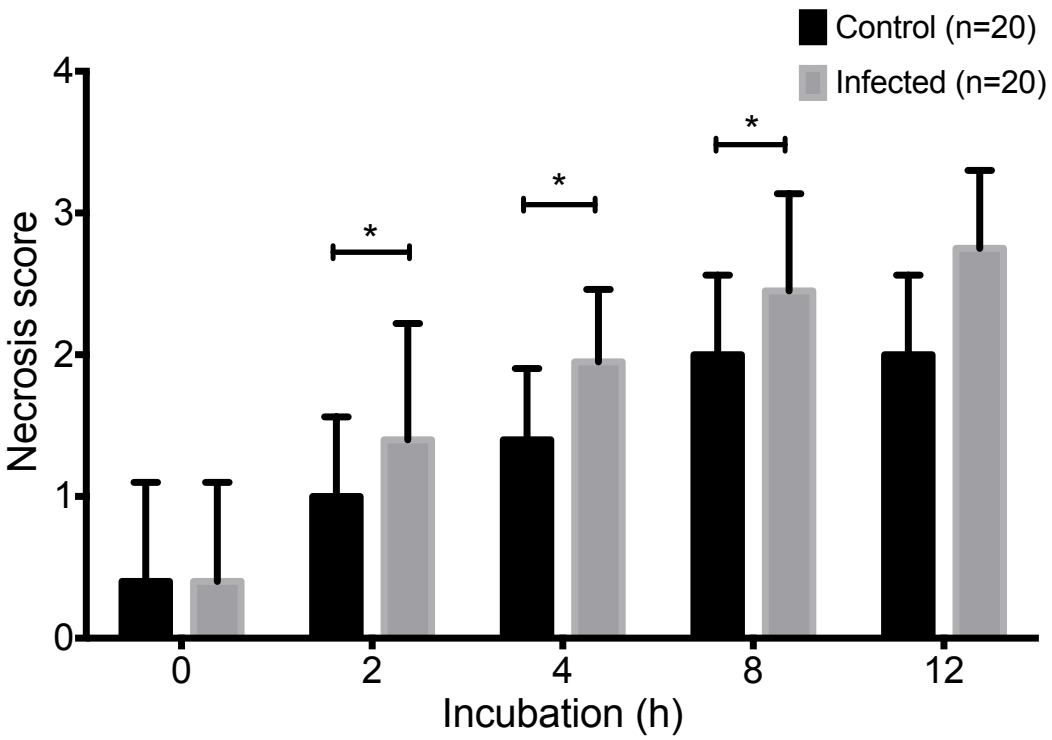

Figure 6.2. Necrosis scores for explants over 12 hours of culture.

Infected group received live *"B. hampsonii"* cells, while control group explants had sterile broth inoculated on the apical side. Bars represent mean necrosis scores from H&E stained sections for 10 pigs, with 2 explants evaluated from each pig at each time point for each group. Stars denote statistically significant differences between groups within time point ($P<0.05$, GEE).

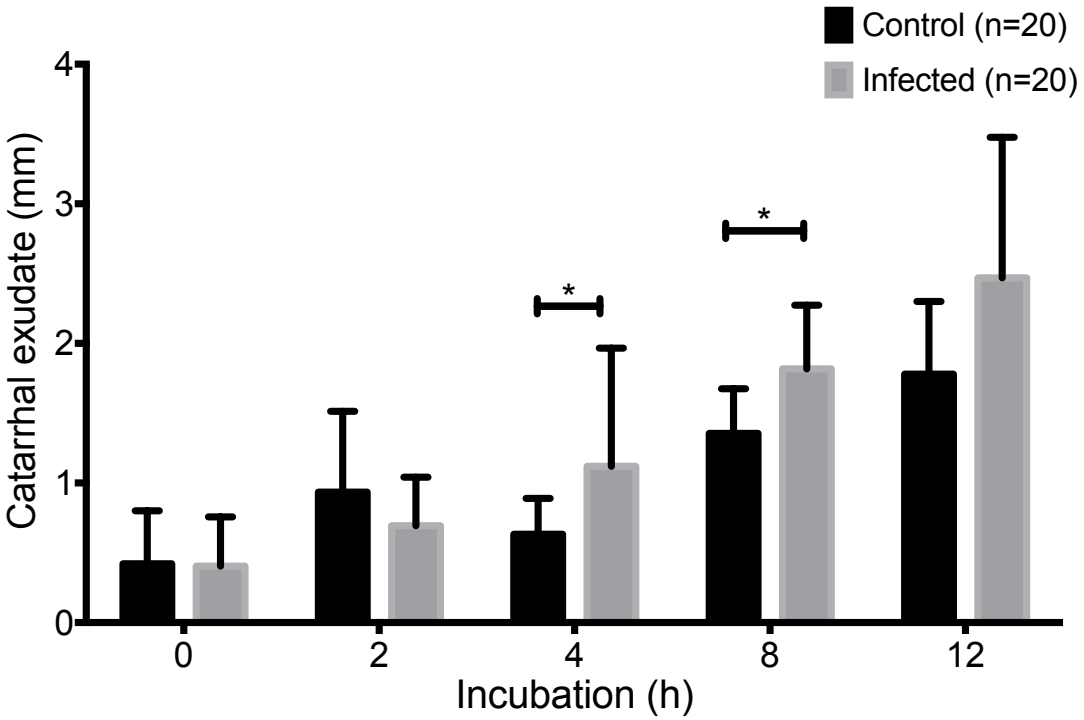

Figure 6.3. Mean catarrhal exudate thickness from explants cultured for 12 hours.

Exudate thickness was measured in five locations along the total explant length. Reported values for each group at a given time represent the mean of two explants cultured from 10 different pigs. Stars denote statistically significant differences between groups within time point ($P<0.05$).

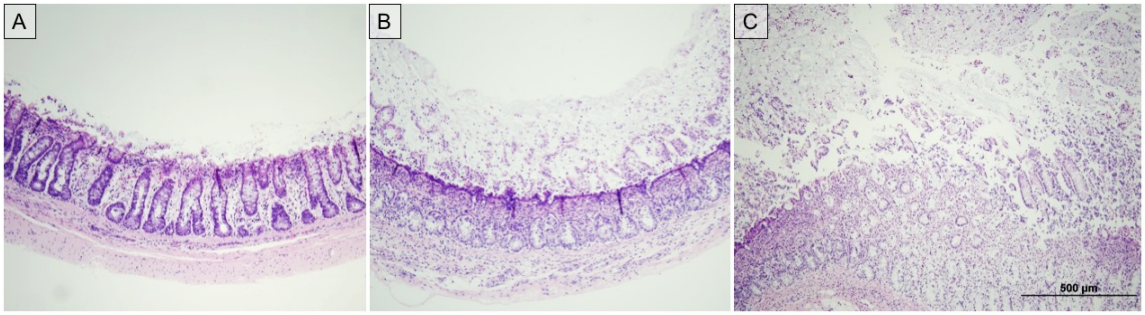

Figure 6.4. Exudate and necrosis in H&E stained explant sections.

A. Typical section of control explant (necrosis score 0) showing very mild catarrhal exudate (Pig #3, 12 hours). B. Infected explant necrosis score 3 and remarkable catarrhal exudate (Pig #1, 12 hours.) C. Infected explant with severe necrosis (score 3) and catarrhal exudate. All photographs taken at 10x.

The following samples were removed from the gene expression analysis since they deviated by more than three standard deviations from the dataset mean: pig #8, control group, 8 hours (e-cadherin), pig #1, control group, 2 hours (IFN-γ), pig #1, infected group, 12 hours (IL-1α), pig #4, control group, 8 hours (IL-8), pig #2, infected group, 12 hours and pig #1, infected group, 4 hours (TNF-α). For all genes, ΔCq values (Cq-target minus Cq GAPDH) decreased from 0-12 hours, indicating higher levels of target gene mRNA relative to GAPDH over time. E-cadherin mRNA levels in the infected group were marginally higher than the control group when all post-infection time points were considered together (P=0.07, GEE), but no significant differences were detected at specific time points. IFN-γ mRNA levels between groups showed a trend of being higher in infected explants (P=0.09, GEE) when all post-infection time points were considered together. IL-1α, IL-8 and TNF-α mRNA levels did not differ between groups. A summary of gene expression data is presented in Figure 6.5.

Localization of spirochaetes within explants was investigated by optical microscopy using Warthin-Faulkner staining. The majority of infected explants had spirochaetes on the apical surface only (score 1, 87%, 72/83), while 10% (8/83) had small numbers of spirochaetes in multiple glands (score 2), 2% (2/83) had no visible spirochates (score 0) and 1% (1/83) had many spirochaetes within several glands (score 3). All control explant sections had no visible spirochates (Table 6.1). Spirochaetes randomly oriented were observed in large numbers in both mucus layers above and in close contact with the epithelium, invading the intercellular space between necrotic and degenerated enterocytes, in end-on association with necrotic epithelial cells and within crypts (Figure 6.6).

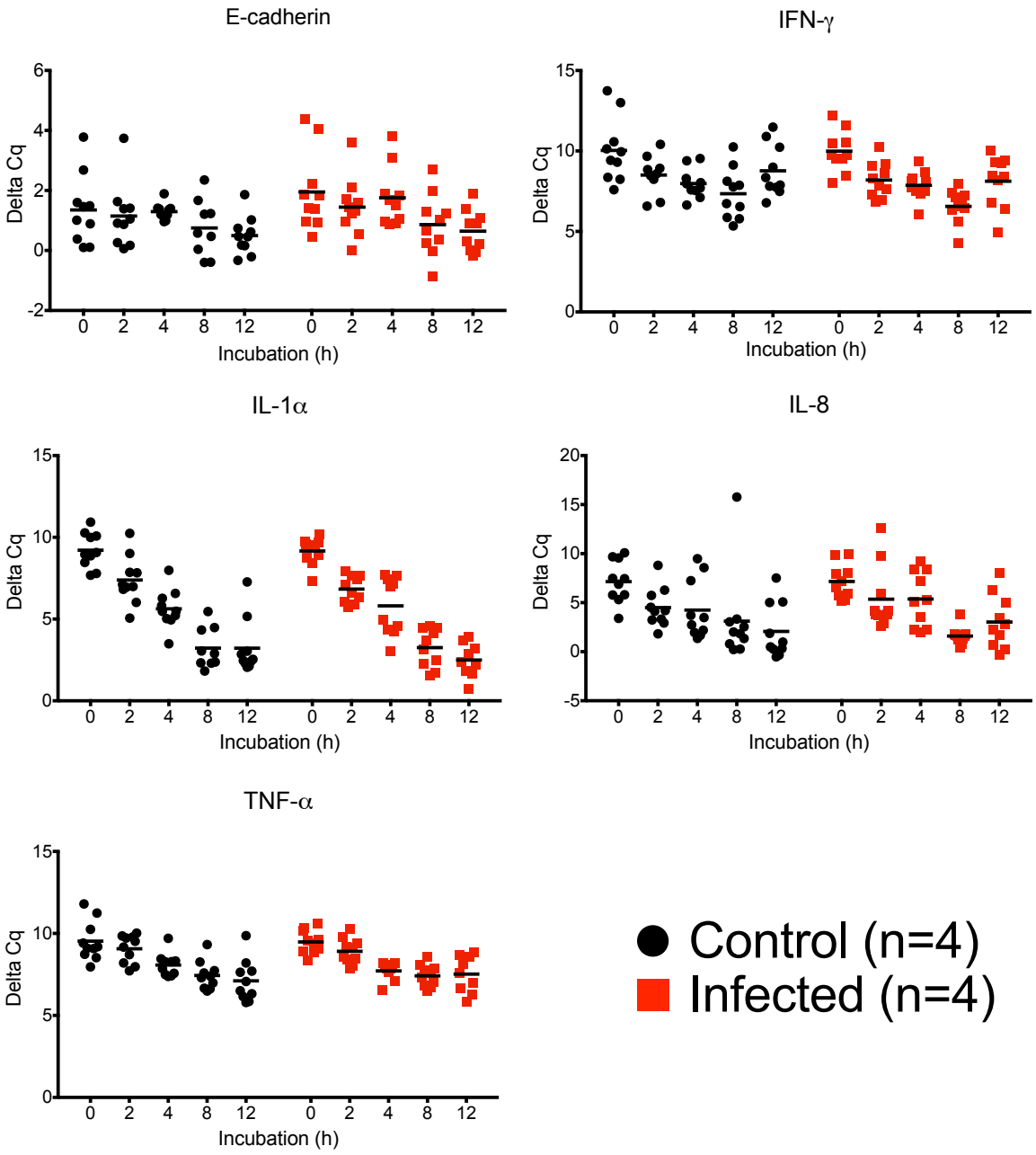

Figure 6.5. ΔCq values of different genes detected in explants over 12 hours of incubation. Each data point represents an average Cq value calculated for one pig. Averages were obtained from four different explants of each pig, run in duplicate reactions. Black bars represent mean ΔCq for each group at a given time point.

Table 6.1. Presence of spirochaetes in Warthin-Faulkner stained explant sections

Group	**No visible spirochaetes (score 0)**	**Visible spirochaetes (score >0)**
Control	100	0
Infected	2	98

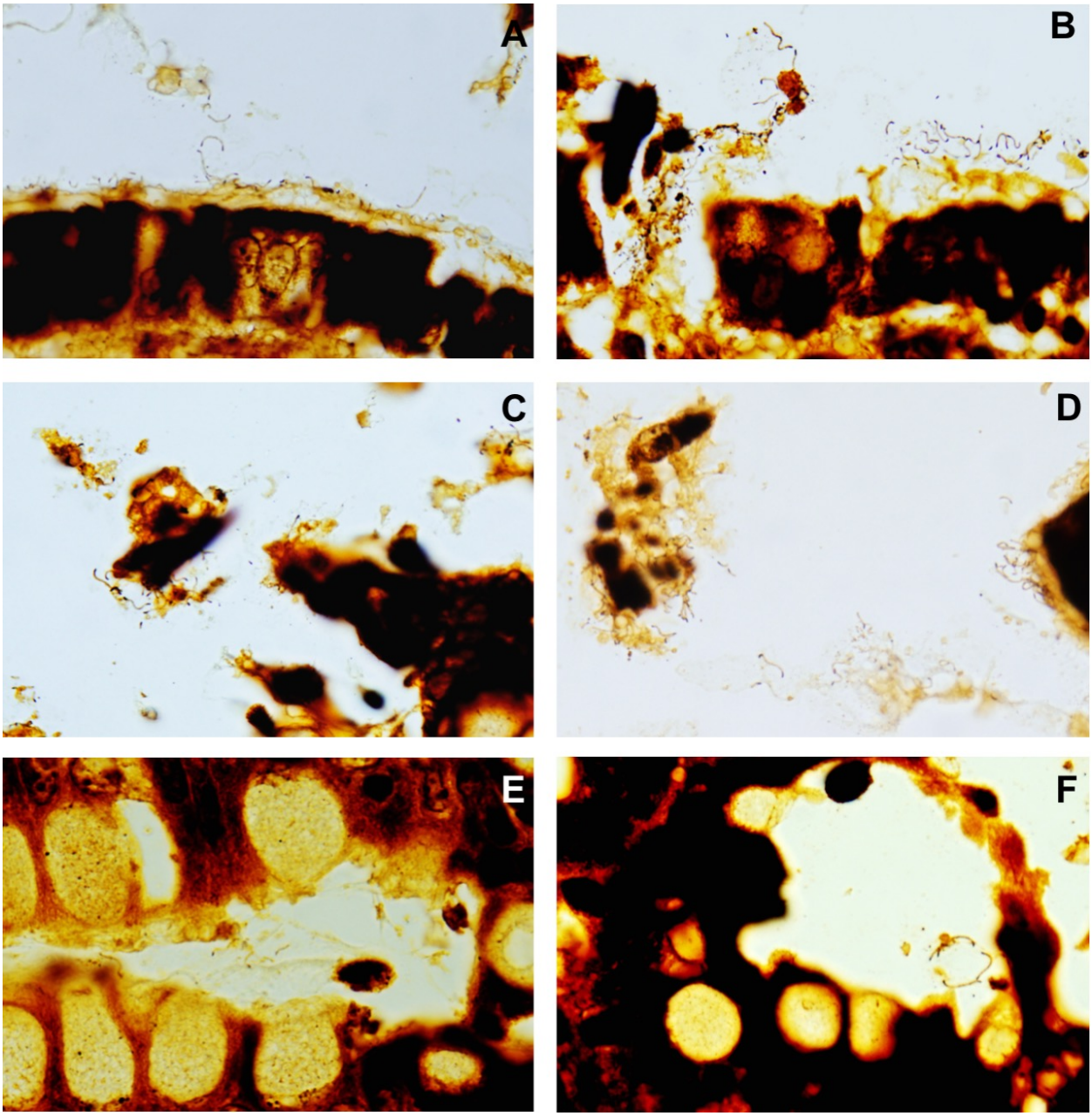

Figure 6.6. Warthin-Faulkner stained sections of explants infected with *"B. hampsonii"*.

A. Pig #5, 4 hours of incubation. Explant surface with randomly oriented, black spirochaetes in the mucus layer immediately above and in close contact with epithelial cells. B. Pig #7, 2 hours

of incubation. Spirochaetes invading the intercellular space left by necrotic epithelial cells. C. Pig #2, 8 hours of incubation. Necrotic cells covered by spirochaetes. D. Pig #6, 8 hours of incubation. Extruded necrotic epithelial cell surrounded by spirochaetes (left side) and spirochaetes in close contact with explant epithelial cells (right side). E. Pig #7, 2 hours of incubation. Explant crypt invaded by a small number of spirochaetes. F. Pig #9, 4 hours of incubation. Necrotizing explant crypt invaded by spirochaetes.

6.5 Discussion

No studies published to date have reported the interaction of pathogenic *Brachyspira* and fully differentiated porcine colon mucosal tissues *in vitro*. Thus, the pathogenesis of the disease associated with *"B. hampsonii"* or *B. hyodysenteriae* is yet to be fully described. To date, a majority of studies have employed *in vivo* pig models to study the disease and have been focused on verification of strain pathogenicity to pigs and clinical and pathological description of the disease (Burrough et al., 2012b; Costa et al., 2014; Harris et al., 1972; Rubin et al., 2013a). In these cases the pig model was ideal as it is the host of interest studied in a controlled environment. However, the fastidious growth of *Brachyspira* and variability in host susceptibility makes it challenging to study the critical period of initial host-pathogen interaction. In this study we exposed porcine distal colon explants to *"B. hampsonii"* clade II strain 30446, which is known to be pathogenic to pigs. Our data demonstrate an interaction between host and pathogen as both partners responded to the co-incubation.

In vitro organ culture models have been previously used to explore interactions of *Shigella* spp., *E. coli*, and *Salmonella* with swine explants, and *Brachyspira pilosicoli* with chicken explants (Collins et al., 2010; Haque et al., 2004; Lembo-Fazio et al., 2011; Mappley et al., 2011; Schüller et al., 2004). However, IVOCs have some relevant limitations when used as host models. It is difficult to quantify and identify all cell populations within an explant; there are limitations to the number of cultured samples based on the donor species and organ; it requires more skilful manipulation of samples than cell cultures, and survival rates of explants are generally poor requiring more replicates to account for losses (Chapter 5). In addition, explants lack blood

circulation, do not allow for interaction with deep tissue, there is no linkage with other organs, no luminal movements due to peristalsis, the indigenous microbiota is controlled by antibiotics in the media, and the organ is subject to stressors from euthanasia or the biopsy sampling process. Conversely, organ cultures allow for controlled infection of the target organ, ease of sampling and great flexibility in experimental designs. Equally important, explants are histologically similar to *in vivo* tissue, retaining structures that cell cultures cannot replicate. In the specific case of swine-associated *Brachyspira*, colonic crypts are consistently described as the main site of spirochaete colonization *in vivo*, usually populated with large numbers of bacterial cells (Costa et al., 2014; Rubin et al., 2013a). Colon explants maintain crypts when *in vitro*, allowing investigation of early host-pathogen interaction.

Our data showed that explants infected with *"B. hampsonii"* had more necrotic cells within crypts and greater catarrhal exudate thickness than the control explants. *In vivo*, *"B. hampsonii*" causes superficial mucosal necrosis, surface accumulation of neutrophils, mucosal crypt elongation, goblet cell hyperplasia and excessive mucus in samples collected from pigs 4 to 14 days after inoculation (Burrough et al., 2012b; Rubin et al., 2013a). Results from an experimental inoculation of pigs with *B. hyodysenteriae* performed by Albassam et al. (1985) suggested that the earliest lesion detectable before the onset of diarrhea was intercellular mucosal edema, which was only visible by electron microscopy. The same authors demonstrated that early lesions detectable by light microscopy were characterized by degeneration, necrosis and extrusion of superficial epithelial cells once pigs began shedding spirochaetes in their feces (Albassam et al., 1985). Despite the difference in time frame, the *in vitro* model used in this study produced similar results to *in vivo* models. Explants developed crypt necrosis similar to

what is seen *in vivo* due to the colonization of the colon by *"B. hampsonii",* suggesting an interaction between host and pathogen. The accumulation of mucus on the apical side of explants from both groups implies that epithelial and goblet cells were actively responding to the culture environment, preventing tissue dehydration. In the specific case of *Brachyspira*, excessive mucus production and catarrhal exudate are key signs observed *in vivo* that were also observed in the IVOC model. Findings like edema and immune cell infiltrates would not be seen in this system since there is no blood supplying explants.

We observed a marginal increase in amounts of e-cadherin mRNA in explants infected with *"B. hampsonii*" relative to controls. E-cadherin is an important part of the adherens junction complex and disturbance of this system may lead to weakened intercellular adherence (Takeichi, 1991). Other enteric pathogens such as *Bacteroides fragilis* and *Salmonella* Typhimurium are associated with increased degradation of e-cadherin protein (Duan et al., 2007; Riegler et al., 1999). To maintain epithelial integrity, a compensatory increase in expression of the e-cadherin is required. Further investigation of the effect of *"B. hampsonii"* on e-cadherin expression using a larger sample size is suggested.

Investigation of SD pigs has shown that infection results in an increased expression of the gene encoding the gel-forming mucin MUC5AC, stimulated by IL-1β and IL-17 (Fujisawa et al., 2009; Wilberts et al., 2014). Based on these *in vivo* observations, we anticipated a difference in IL-1α expression between groups that was not observed. Our choice to target IL-1α, instead of IL-1β, was based on the fact that the IL-1α precursor molecule is an important damage-associated molecular pattern protein (or *alarmin*), while the IL-1β precursor is biologically

inactive (Cohen et al., 2010). Thus, IL-1α also acts as an early tissue-damage signalling molecule, fit for the short (12 hour) incubation period of this trial. Kruse et al. reported that experimental infection of pigs with *B. hyodysenteriae* led to increased serum levels of IL-1β and TNF-α during peak clinical signs, while there were undetectable amounts of IFN-γ before and after inoculation (Kruse et al., 2008). It is noteworthy that cytokine production is different between organs and serum levels may not reflect a localized or early inflammatory response. We observed a trend toward higher levels of IFN-γ mRNA over the 12 hour period in infected explants relative to control (Figure 6.4). Such a local response may be important as a first host response to the pathogen, possibly acting *in vivo* as a chemotactic, recruiting innate response cells from circulation and preventing further invasion of the colon.

We did not observe any differences in IL-8 between groups. In a human colonic epithelial cell model, live *B. pilosicoli* cells and culture sonicate induced the up-regulation of IL-1β and IL-8 after 12 hours of incubation, as well as disruption of epithelial zonula occludens (Naresh et al., 2009). *B. pilosicoli* is associated with a mild diarrheic syndrome in pigs. Microscopically, during early stages of infection, *B. pilosicoli* is found attached to colonic epithelial cells, a phenomenon described as "false brush-border" (Muniappa et al., 1998; Naresh et al., 2009). Results of the current study agree with previous reports that "*B. hampsonii"* does not attach or invade beyond the colonic epithelial cell layer. This difference in host-pathogen intimacy may contribute to activation of different inflammatory pathways, culminating in different cytokine expression patterns.

Colon sections of pigs with SD show spirochaetes within crypts, between colonic epithelial cells, free within the cytoplasm of enterocytes and in the lamina propria (Albassam et al., 1985; Jensen et al., 1998; Taylor and Blakemore, 1971). *"B. hampsonii"* has been reported to colonize the colon similarly to *B. hyodysenteriae*, but to date no description of *"B. hampsonii"* within the lamina propria has been made (Burrough et al., 2012b; Rubin et al., 2013a). Similar to *in vivo* infection by either pathogen, we observed spirochaetes within the mucus layer immediately above epithelial cells, between necrotic and degenerated enterocytes and within crypts. We also described a more intense extrusion of epithelial necrotic cells from infected explants than control explant. Taken together, our data suggest that IVOCs are recognized by the spirochaetes as a host environment. Currently, it is unknown if there is a need for spirochaete attachment by receptor:ligand interaction with enterocytes to cause disease. In addition, there is no description in the literature of one or more enterotoxins produced by *B. hyodysenteriae* or *"B. hampsonii"* associated with the microscopic lesions observed when live spirochates are present in the colon. This may be one of the biggest mysteries surrounding *Brachyspira*-associated disease in pigs, as the tissue-damage mechanisms remain to be understood.

6.6 Conclusions

This study demonstrated that pig colon explants are recognized by *"B. hampsonii"* as a target, leading to increased cellular death and increased thickness of the catarrhal exudate layer above the mucosa. In addition, we described similar spirochaetal colonization patterns of explants to what is observed *in vivo*. Cytokine response to the pathogen was unremarkable, with no significant differences observed in expression of the studied genes. Taken together, these results

indicate that this novel *in vitro* infection model performs satisfactorily for "*B. hampsonii"* investigations, adds to the available evidence that "*B. hampsonii*" is pathogenic to pigs and suggests that the cytokine response during early infection periods is different from the response at peak clinical signs. In future work, investigation of expression of additional cytokines, apical junction proteins and different time points is suggested to help clarify the microscopic lesions described.

7 General Discussion

7.1 Summary and limitations of these works

7.1.1 "Brachyspira hampsonii" clades I and II cause mucohaemorrhagic diarrhea in pigs

Classically, the etiologic agent associated with mucohaemorrhagic diarrhea and colitis in grower-finisher pigs was *B. hyodysenteriae* (Harris et al., 1972). Until the mid-2000s, swine veterinarians and producers were accustomed to this association and the available diagnostic methods for *Brachyspira* supported their assumption. At that time, pathogenic strains were identified by their ability to completely haemolyse erythrocytes in blood agar plates. Thus, strong β-haemolytic isolates from clinical cases were reported as *B. hyodysenteriae* due to the lack of more specific tests. The application of molecular biology techniques to diagnostics increased the resolution of the tests, enabling the detection and identification of "atypical" *Brachyspira* associated with bloody diarrhea, such as "*B. hampsonii"*.

The results presented in this thesis have established the causal relationship between the novel *"B. hampsonii"* and mucohaemorrhagic colitis and diarrhea in pigs. Regarding Chapters 2 & 3, it is important to recognize that small population sizes were used in the inoculation studies. While beneficial from an animal welfare perspective, descriptive aspects of the study such as gross and microscopic pathology could benefit if more animals were used in the study. In addition, investigators were not blinded to treatments throughout the trial. Despite these limitations, we were able to characterize the disease. Another concerning aspect was the detection of *"B.*

hampsonii" and other *Brachyspira* spp. in pigs prior to inoculation. This previous exposure to spirochates may have led to development of a mucosal immune response to *"B. hampsonii"* that perhaps was reflected in the proportion of inoculated pigs that did not develop bloody diarrhea in both clade I and II trials. It also raises questions regarding susceptibility factors associated with the disease.

7.1.2 Colon infection by "B. hampsonii" leads to a shift in the microbiota composition

Susceptibility factors associated with mucohaemorrhagic diarrhea in pigs infected with *Brachyspira* have not been completely clarified. Development of the disease is suggested to be multifactorial, not solely dependent on spirochaete colonization of the colon (Alvarez-Ordonez et al., 2013). For example, there is evidence that gnotobiotic pigs inoculated with pure cultures of *B. hyodysenteriae* do not develop mucohaemorrhagic diarrhea as seen in conventionally raised pigs (Harris et al., 1978; Whipp et al., 1982). We have shown that the composition of the fecal microbial communities of diarrheic pigs were different from healthy pigs, whether they were inoculated or not. However, we did not identify a characteristic microbiome profile indicative of susceptibility to infection. These findings are similar to what other authors reported when investigating *Salmonella* infection and its effect on the microbiome (Bearson et al., 2013). Similar to our report, these authors also observed how only pigs shedding significant amounts of *Salmonella* had their microbiome profile disturbed, while low-shedders and controls remained indistinguishable. In fact, the difference was due to lower abundance of bacteria from the phylum Bacteroidetes in pigs sheddinge significant amounts of *Salmonella*. It is not a surprise that the

onset of clinical diarrhea disturbs the microbiome composition to a significant level, given the pathophysiological changes that occur due to the disease process. This has been observed by other authors in rodents, dogs and swine (Chaban et al., 2010; Koh et al., 2015; Lupp et al., 2007).

We recognize that a small sample size was used in this study and perhaps it prevented us from identifying a microbiome profile that would render pigs more susceptible to infection by *"B. hampsonii"*. We also recognize that despite the reported values for Good's coverage falling within acceptable ranges, this study could benefit from greater sequencing depth and that could be potentially revealing in terms of shifts associated with disease susceptibility.

7.1.3 In vitro culture of swine colon reveals maintenance of histological characteristics similar to in vivo after 5 days of incubation

Results from this study demonstrated that *in vitro* organ culture methods can support viable pig colon explants for 5 days. Explant sections showed typical colon structures such as columnar epithelium and crypts. The study also revealed significant variation in explant survivability between explants from the same donor. It is important to recognize that poor reproducibility appears to be an aspect of this technique that has been observed previously (Reiss and Williams, 1979). Many factors may affect each explant, from proximity to ice during transport to mechanical strength applied to the tissue during handling, and all of these factors contribute to tissue survival *in vitro*.

Development and standardization of a protocol that successfully supported *ex vivo* culture of colonic tissue required the analysis of different factors possibly associated with explant survivability. We evaluated the age of pigs at euthanasia, the donor colon segment, transport time to the laboratory, the media supplements and the time in culture. Given the availability of animals, multiple trials were conducted at different times to evaluate all the factors. It is important to recognize that there was considerable variation between trials, as pigs differed in farm of origin, genetic lines, exposure to stressful situations and handling. In addition, due to the extended time required to perform all the trials, reagents did not belong to the same lots throughout all studies. While adding variation to the study, it simulates what other investigators may face when using this protocol.

It is not unrealistic to suggest that porcine colon explants can be cultured for longer periods than we described and yet retain good histological features. During pilot experiments, we anecdotally observed explants cultured for 9 days with intact crypts and columnar epithelium. However, poor explant survival was seen overall. Suggested modifications to the protocol include the use of a rocking platform to prevent the accumulation of mucus, enzymes, secreted hormones and growth factors within the mucosa. *De facto*, a thick mucus layer visible to the naked eye is seen within the first 12 hours of culture on the mucosal side of explants. A rocking platform would act to simulate the peristaltic movements of the colon, washing the mucus from the explants and allowing for better diffusion of oxygen to the tissue. In addition, media from cultures under these conditions would become an interesting source of metabolites for analysis, as it constantly washes the explants. The agar attachment matrix might also be exchanged for synthetic materials. Pilot experiments using necropsy foam to support explants were performed (data not

shown). Necropsy foam has relatively massive visible pores, allowing for diffusion of media to the explant. In these pilot experiments, good quality explants were observed after 24 hours of culture. However, the foam pieces floated freely in the media as "rafts" with explants on top, which would pose a problem when polarizing the system for inoculation of the mucosal side. Nutritionally, it is hypothesized that the supplementation of explants with volatile fatty acids would contribute to maintenance of crypt depth and epithelial characteristics.

The porcine colon IVOC system developed has a lot of potential applications, and modifications to the described protocol are strongly encouraged by the authors.

7.1.4 "B. hampsonii" colonizes the mucus layer, inducing necrosis and excessive exudate from swine colon explants

Mucohaemorrhagic diarrhea in pigs has been associated with *Brachyspira* spp. for over 40 years (Harris et al., 1972). However, an important knowledge gap still remains regarding the mechanisms employed by *B. hyodysenteriae*, and now also by *"B. hampsonii,* that result in severe enteric disease. To address this gap, an *in vitro* infection model was used to investigate the first 12 hours of interaction between *"B. hampsonii"* and colon explants. Lesions were demonstrated in infected explants, although no clear inflammatory response was observed at the gene expression level. The observation of increased exudate thickness and increased number of necrotic cells within crypts are similar to *in vivo* descriptions of microscopic lesions. However, the number spirochaetes found within glands was relatively small when compared to what was

described in our *in vivo* infection trials. The formalin fixation process may contribute to this observation, since mucus and cells may be washed away from explant surfaces and crypts when the tissue sections are immersed in the fixative. Future improvements to the sample collection method may incorporate a more gentle fixation process, including immersion of samples in an alternative fixation solution such as Carnoy's solution that would do a better job of preserving the mucus layer (Matsuo et al., 1997). Despite these limitations, results of the IVOC inoculation trial clearly demonstrate histopathological changes characteristic of a biological response by the colon explants to pathogen exposure.

Previous studies in pigs infected with *B. hyodysenteriae* demonstrated increased serum levels of IL-1β and TNFα before and during peak clinical signs (Kruse et al., 2008). Based on this *in vivo* report, we anticipated that expression of acute pro-inflammatory cytokines would be upregulated upon exposure of explants to *"B. hampsonii"*. Despite a marginally significant increase in IFN-γ levels, expression of pro-inflammatory cytokines by explants over the study period was unremarkable. Perhaps there was an inflammatory response by the epithelium, but the remarkably greater number of cells in the lamina propria that were unaffected masked that response. It is also possible that the 2-12 hour study period was not long enough to observe the expected cytokine response. Conversely, the inflammatory response may have happened between 0 and 2 hours post-infection and sampling two hours after the initial exposure may have been too late. Another explanation is that an inflammatory response happened but was not related to the genes selected for this study. Based on *in vivo* reports, gene expression analyses of IL-1β and IL-6 are suitable alternatives. Ultimately, it is also possible that the IVOC protocol developed

renders explants immunologically unresponsive to "*B. hampsonii"*. The aspects discussed above should be considered when designing future experiments.

Although no statistically significant difference was detected, a trend towards increased expression of e-cadherin by infected explants was observed. This suggests a compensatory response due to increased e-cadherin protein degradation. A greater number of explants and pigs may be necessary to further verify this finding. Decreased levels of e-cadherin protein, an important intercellular adhesion glycoprotein, could be related to degradation of the structural integrity of the epithelium by the pathogen. If true, this finding would contradict the conclusions of Argenzio et al. that *B. hyodysenteriae* causes malabsorptive diarrhea, unrelated to increased mucosal permeability or leakage (Argenzio et al., 1980). Further investigation of the effects of *Brachyspira* infection on the expression of e-cadherin and other factors affecting epithelium barrier function such as zonula occludens 1, claudin-1 an claudin-3 is clearly warranted.

7.2 Discussion of future prospects

7.2.1 Understanding the pathogenesis of mucohaemorrhagic diarrhea in pigs is a key step towards disease control

Investigations conducted in this thesis resulted in the confirmation that the novel "*B. hampsonii"* causes mucohaemorrhagic diarrhea in pigs. The latter chapters of this work focused on a primary aspect of preventive veterinary medicine: understanding disease pathogenesis. In the particular case of *"B. hampsonii"* there is a remarkable knowledge gap regarding host-pathogen

interactions. It is still unknown how the spirochaetes interact with the host: Is there cell-to-cell attachment? Is spirochaete attachment necessary for development of lesions? We still do not completely understand how lesions develop: Are there toxins produced by the bacteria? What are they? Do haemolysins play a role *in vivo*? It is not imprudent to state that despite the description of the disease almost 100 years ago, there is more unknown than known about mucohaemorrhagic colitis and diarrhea in pigs caused by *Brachyspira* spp..

The IVOC protocol described in this thesis is an attempt to model the disease, which may help clarify many of the questions stated above. This first step was taken to show that *"B. hampsonii"* interacts with the explants *in vitro*. However, further characterization of this interaction is needed. Is there modulation of absorptive pathways in epithelial cells? Is the epithelial barrier disrupted? What role does the apical junction complex play in the development of the disease, if any? What mucin genes are upregulated to produce the observed excessive catarrhal exudate? How does *"B. hampsonii"* modulate that response? These are some of the questions that remain unanswered from the host perspective of the infection. In parallel, the IVOC model should also be applied to understand the pathogen response after exposure to the host. What triggers the expression of virulence factors? Why do some pigs not show lesions despite being inoculated? What role do goblet cells play in pathogen stimulation? Why does *Brachyspira* infection apparently elicit a weak mucosal response?

There are important questions that future efforts may approach, when it comes to "*B. hampsonii"* pathogenesis, and answering any or all of them will contribute to the common goal of developing better tools for prevention of infection with this pathogen.

8 References

Abbott, A., 2003. Cell culture: biology's new dimension. Nature 424, 870-872.

Abud, H.E., Watson, N., Heath, J.K., 2005. Growth of intestinal epithelium in organ culture is dependent on EGF signalling. Experimental Cell Research 303, 252-262.

Adams, R.B., Planchon, S.M., Roche, J.K., 1993. IFN-gamma modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding. Journal of Immunology 150, 2356-2363.

Akase, S., Uchitani, Y., Sohmura, Y., Tatsuta, K., Sadamasu, K., Adachi, Y., 2009. Application of real-time PCR for diagnosis of Swine Dysentery. The Journal of Veterinary Medical Science 71, 359-362.

Albassam, M.A., Olander, H.J., Thacker, H.L., Turek, J.J., 1985. Ultrastructural characterization of colonic lesions in pigs inoculated with *Treponema hyodysenteriae*. Canadian Journal of Comparative Medicine 49, 384-390.

Alvarez-Ordonez, A., Martinez-Lobo, F.J., Arguello, H., Carvajal, A., Rubio, P., 2013. Swine dysentery: aetiology, pathogenicity, determinants of transmission and the fight against the disease. International Journal of Environmental Research and Public Health 10, 1927-1947.

Antoni, L., Nuding, S., Weller, D., Gersemann, M., Ott, G., Wehkamp, J., Stange, E.F., 2013. Human colonic mucus is a reservoir for antimicrobial peptides. Journal of Crohn's and Colitis 7, e652-664.

Appleton, G.V., Wheeler, E.E., Challacombe, D.N., Williamson, R.C., 1991. Validation of organ culture in colonic adaptation to surgical manipulation. Gut 32, 1027-1030.

Arena, E.T., Campbell-Valois, F.X., Tinevez, J.Y., Nigro, G., Sachse, M., Moya-Nilges, M., Nothelfer, K., Marteyn, B., Shorte, S.L., Sansonetti, P.J., 2015. Bioimage analysis of

Shigella infection reveals targeting of colonic crypts. Proceedings of the National Academy of Sciences 112, E3282-3290.

Argenzio, R.A., 1980. Glucose-stimulated fluid absorption in the pig small intestine during the early stage of swine dysentery. American Journal of Veterinary Research 41, 2000-2006.

Argenzio, R.A., Whipp, S.C., Glock, R.D., 1980. Pathophysiology of swine dysentery: colonic transport and permeability studies. Journal of Infectious Diseases 142, 676-684.

Asker, N., Axelsson, M.A., Olofsson, S.O., Hansson, G.C., 1998. Dimerization of the human MUC2 mucin in the endoplasmic reticulum is followed by a N-glycosylation-dependent transfer of the mono and dimers to the Golgi apparatus. Journal of Biological Chemistry 273, 18857-18863.

Atyeo, R.F., Stanton, T.B., Jensen, N.S., Suriyaarachichi, D.S., Hampson, D.J., 1999. Differentiation of *Serpulina* species by NADH oxidase gene (nox) sequence comparisons and nox-based polymerase chain reaction tests. Veterinary Microbiology 67, 47-60.

Autrup, H., Barrett, L.A., Jackson, F.E., Jesudason, M.L., Stoner, G., Phelps, P., Trump, B.F., Harris, C.C., 1978a. Explant culture of human colon. Gastroenterology 74, 1248-1257.

Autrup, H., Stoner, G.D., Jackson, F., Harris, C.C., Shamsuddin, A.K., Barrett, L.A., Trump, B.F., 1978b. Explant culture of rat colon: a model system for studying metabolism of chemical carcinogens. In Vitro 14, 868-877.

Bandaletova, T., Bailey, N., Bingham, S.A., Loktionov, A., 2002. Isolation of exfoliated colonocytes from human stool as a new technique for colonic cytology. APMIS 110, 239-246.

Bartnik, W., ReMine, S.G., Chiba, M., Thayer, W.R., Shorter, R.G., 1980. Isolation and characterization of colonic intraepithelial and lamina proprial lymphocytes. Gastroenterology 78, 976-985.

Bearson, S.M., Allen, H.K., Bearson, B.L., Looft, T., Brunelle, B.W., Kich, J.D., Tuggle, C.K., Bayles, D.O., Alt, D., Levine, U.Y., Stanton, T.B., 2013. Profiling the gastrointestinal microbiota in response to *Salmonella*: Low versus high *Salmonella* shedding in the natural porcine host. Infection, Genetics and Evolution 16C, 330-340.

Bellgard, M.I., Wanchanthuek, P., La, T., Ryan, K., Moolhuijzen, P., Albertyn, Z., Shaban, B., Motro, Y., Dunn, D.S., Schibeci, D., Hunter, A., Barrero, R., Phillips, N.D., Hampson, D.J., 2009. Genome sequence of the pathogenic intestinal spirochete *Brachyspira hyodysenteriae* reveals adaptations to its lifestyle in the porcine large intestine. PLoS One 4, e4641.

Benos, D.J., Cunningham, S., Baker, R.R., Beason, K.B., Oh, Y., Smith, P.R., 1992. Molecular characteristics of amiloride-sensitive sodium channels. Reviews of Physiology, Biochemistry and Pharmacology 120, 31-113.

Best, A., La Ragione, R.M., Clifford, D., Cooley, W.A., Sayers, A.R., Woodward, M.J., 2006. A comparison of *Shiga*-toxin negative *Escherichia coli* O157 aflagellate and intimin deficient mutants in porcine *in vitro* and *in vivo* models of infection. Veterinary Microbiology 113, 63-72.

Binek, M., Szynkiewicz, Z.M., 1984. Physiological properties and classification of strains of *Treponema* sp. isolated from pigs in Poland. Comparative Immunology, Microbiology & Infectious Diseases 7, 141-148.

Bishara, J., Farah, R., Mograbi, J., Khalaila, W., Abu-Elheja, O., Mahamid, M., Nseir, W., 2013. Obesity as a risk factor for *Clostridium difficile* infection. Clinical Infectious Diseases 57, 489-493.

Bohnhoff, M., Drake, B.L., Miller, C.P., 1954. Effect of streptomycin on susceptibility of intestinal tract to experimental Salmonella infection. Proceedings of The Society for Experimental Biology and Medicine 86, 132-137.

Bonini, P., Plebani, M., Ceriotti, F., Rubboli, F., 2002. Errors in laboratory medicine. Clinical Chemistry 48, 691-698.

Bonnet, R., 1895. Uber die "Schlussleisten" der Epithelium. Deutsche Medizinische Wochenschrift 21, 58.

Borowitz, D., Durie, P.R., Clarke, L.L., Werlin, S.L., Taylor, C.J., Semler, J., De Lisle, R.C., Lewindon, P., Lichtman, S.M., Sinaasappel, M., Baker, R.D., Baker, S.S., Verkade, H.J., Lowe, M.E., Stallings, V.A., Janghorbani, M., Butler, R., Heubi, J., 2005. Gastrointestinal outcomes and confounders in cystic fibrosis. Journal of Pediatric Gastroenterology and Nutrition 41, 273-285.

Brandtzaeg, P., Bjerke, K., Kett, K., Kvale, D., Rognum, T.O., Scott, H., Sollid, L.M., Valnes, K., 1987. Production and secretion of immunoglobulins in the gastrointestinal tract. Annals of Allergy, Asthma & Immunology 59, 21-39.

Brooke, C.J., Riley, T.V., Hampson, D.J., 2003. Evaluation of selective media for the isolation of *Brachyspira aalborgi* from human faeces. Journal of Medical Microbiology 52, 509-513.

Browning, T.H., Trier, J.S., 1969. Organ culture of mucosal biopsies of human small intestine. Journal of Clinical Investigation 48, 1423-1432.

Bruewer, M., Luegering, A., Kucharzik, T., Parkos, C.A., Madara, J.L., Hopkins, A.M., Nusrat, A., 2003. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. Journal of Immunology 171, 6164-6172.

Brunsgaard, G., 1997. Morphological characteristics, epithelial cell proliferation, and crypt fission in cecum and colon of growing pigs. Digestive Diseases and Sciences 42, 2384-2393.

Budinger, M.E., Foster, E.S., Hayslett, J.P., Binder, H.J., 1986. Sodium and chloride transport in the large intestine of potassium-loaded rats. American Journal of Physiology 251, G249-252.

Bull, D.M., Bookman, M.A., 1977. Isolation and functional characterization of human intestinal mucosal lymphoid cells. Journal of Clinical Investigation 59, 966-974.

Bullwinkel, J., Baron‐Lühr, B., Lüdemann, A., Wohlenberg, C., Gerdes, J., Scholzen, T., 2006. Ki‐67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. Journal of Cellular Physiology 206, 624-635.

Burrough, E., Strait, E., Kinyon, J., Bower, L., Madson, D., Schwartz, K., Frana, T., Songer, J.G., 2012a. Comparison of atypical *Brachyspira* spp. clinical isolates and classic strains in a mouse model of swine dysentery. Veterinary Microbiology 160, 387-394.

Burrough, E.R., Strait, E.L., Kinyon, J.M., Bower, L.P., Madson, D.M., Wilberts, B.L., Schwartz, K.J., Frana, T.S., Songer, J.G., 2012b. Comparative virulence of clinical *Brachyspira* spp. isolates in inoculated pigs. Journal of Veterinary Diagnostic Investigation 24, 1025-1034.

Butterfield, I., Warhurst, G., Jones, M.N., Sandle, G.I., 1997. Characterization of apical potassium channels induced in rat distal colon during potassium adaptation. Journal of Physiology 501 (Pt 3), 537-547.

Chaban, B., Chu, S., Hendrick, S., Waldner, C., Hill, J.E., 2012. Evaluation of a *Campylobacter fetus* subspecies venerealis real-time quantitative polymerase chain reaction for direct analysis of bovine preputial samples. Canadian Journal of Veterinary Research 76, 166-173.

Chaban, B., Ngeleka, M., Hill, J.E., 2010. Detection and quantification of 14 *Campylobacter* species in pet dogs reveals an increase in species richness in feces of diarrheic animals. BMC Microbiology 10, 73.

Chander, Y., Primus, A., Oliveira, S., Gebhart, C.J., 2012. Phenotypic and molecular characterization of a novel strongly hemolytic *Brachyspira* species, provisionally designated "*Brachyspira hampsonii*". Journal of Veterinary Diagnostic Investigation 24, 903-910.

Chao, A.C., de Sauvage, F.J., Dong, Y.J., Wagner, J.A., Goeddel, D.V., Gardner, P., 1994. Activation of intestinal CFTR Cl- channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase. EMBO Journal 13, 1065-1072.

Chia, S.P., Taylor, D.J., 1978. Factors affecting the survival of *Treponema hyodysenteriae* in dysenteric pig faeces. Veterinary Records 103, 68-70.

Christensen, J., Ellegaard, B., Petersen, B.K., Willeberg, P., Mousing, J., 1994. Pig health and production surveillance in Denmark - sampling design, data recording, and measures of disease frequency. Preventive Veterinary Medicine 20, 47-61.

Christl, S.U., Scheppach, W., 1997. Metabolic consequences of total colectomy. Scandinavian Journal of Gastroenterology 222, 20-24.

Cliff, W.H., Frizzell, R.A., 1990. Separate Cl- conductances activated by cAMP and Ca2+ in Cl(-)-secreting epithelial cells. Proceedings of the National Academy of Sciences 87, 4956-4960.

Clothier, K.A., Kinyon, J.M., Frana, T.S., Naberhaus, N., Bower, L., Strait, E.L., Schwartz, K., 2011. Species characterization and minimum inhibitory concentration patterns of *Brachyspira* species isolates from swine with clinical disease. Journal of Veterinary Diagnostic Investigation 23, 1140-1145.

Cohen, I., Rider, P., Carmi, Y., Braiman, A., Dotan, S., White, M.R., Voronov, E., Martin, M.U., Dinarello, C.A., Apte, R.N., 2010. Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. Proceedings of the National Academy of Sciences 107, 2574-2579.

Collado, M.C., Meriluoto, J., Salminen, S., 2007. Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Letters in Applied Microbiology 45, 454-460.

Collins, J.W., Coldham, N.G., Salguero, F.J., Cooley, W.A., Newell, W.R., Rastall, R.A., Gibson, G.R., Woodward, M.J., La Ragione, R.M., 2010. Response of porcine intestinal *in vitro* organ culture tissues following exposure to *Lactobacillus plantarum* JC1 and *Salmonella enterica* serovar Typhimurium SL1344. Applied Environmental Microbiology 76, 6645-6657.

Compher, C.W., Frankel, W.L., Tazelaar, J., Lawson, J.A., McKinney, S., Segall, S., Kinosian, B.P., Williams, N.N., Rombeau, J.L., 1999. Wheat bran decreases aberrant crypt foci, preserves normal proliferation, and increases intraluminal butyrate levels in experimental colon cancer. Journal of Parenteral and Enteral Nutrition 23, 269-277.

Costa, M.O., Hill, J.E., Fernando, C., Lemieux, H.D., Detmer, S.E., Rubin, J.E., Harding, J.C., 2014. Confirmation that "*Brachyspira hampsonii*" clade I (Canadian strain 30599) causes

mucohemorrhagic diarrhea and colitis in experimentally infected pigs. BMC Veterinary Research 10, 129.

Crost, E.H., Pujol, A., Ladire, M., Dabard, J., Raibaud, P., Carlier, J.P., Fons, M., 2010. Production of an antibacterial substance in the digestive tract involved in colonization-resistance against *Clostridium perfringens*. Anaerobe 16, 597-603.

Dame, M.K., Veerapaneni, I., Bhagavathula, N., Naik, M., Varani, J., 2011. Human colon tissue in organ culture: calcium and multi-mineral-induced mucosal differentiation. *In Vitro* Cellular Developmental Biology - Animal 47, 32-38.

Danielsen, E.M., Sjostrom, H., Noren, O., Bro, B., Dabelsteen, E., 1982. Biosynthesis of intestinal microvillar proteins. Characterization of intestinal explants in organ culture and evidence for the existence of pro-forms of the microvillar enzymes. Journal of Biochemistry 202, 647-654.

Dawson, D.C., 1991. Ion channels and colonic salt transport. Annual Review of Physiology 53, 321-339.

Deschner, E., Lewis, C.M., Lipkin, M., 1963. *In vitro* study of human rectal epithelial cells. I. Atypical zone of h3 thymidine incorporation in mucosa of multiple polyposis. Journal of Clinical Investigation 42, 1922-1928.

Dobbins, W.O., 3rd, Herrero, B.A., Mansbach, C.M., 1968. Morphologic alterations associated with neomycin induced malabsorption. The American Journal of the Medical Sciences 255, 63-77.

Drossman, D.A., Powell, D.W., Sessions, J.T., Jr., 1977. The irritable bowel syndrome. Gastroenterology 73, 811-822.

Duan, Y., Liao, A.P., Kuppireddi, S., Ye, Z., Ciancio, M.J., Sun, J., 2007. Beta-catenin activity negatively regulates bacteria-induced inflammation. Laboratory Investigation 87, 613-624.

Dufresne, L. 1999. Evaluating the economical return of health program. In Allen D. Leman Conference (Saint Paul, MN), 3.

Duhamel, G.E., 2001. Comparative pathology and pathogenesis of naturally acquired and experimentally induced colonic spirochetosis. Animal Health Research Reviews 2, 3-17.

Durmic, Z., Pethick, D.W., Pluske, J.R., Hampson, D.J., 1998. Changes in bacterial populations in the colon of pigs fed different sources of dietary fibre, and the development of swine dysentery after experimental infection. Journal of Applied Microbiology 85, 574-582.

Durso, L.M., Harhay, G.P., Smith, T.P.L., Bono, J.L., DeSantis, T.Z., Harhay, D.M., Andersen, G.L., Keen, J.E., Laegreid, W.W., Clawson, M.L., 2010. Animal-to-animal variation in fecal microbial diversity among beef cattle. Applied and Environmental Microbiology 76, 4858-4862.

Dyce, K.M., Sack, W.O., Wensing, C.J.G., 2009. Textbook of Veterinary Anatomy, 4th Edition. Elsevier Science Health Science Division, Philadelphia.

Eckmann, L., Jung, H.C., Schurer-Maly, C., Panja, A., Morzycka-Wroblewska, E., Kagnoff, M.F., 1993. Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8. Gastroenterology 105, 1689-1697.

Elphick, D.A., Mahida, Y.R., 2005. Paneth cells: their role in innate immunity and inflammatory disease. Gut 54, 1802-1809.

Erickson, H.H., Goff, J.P., Uemura, E.E., Reece, W.O., 2015. Dukes' physiology of domestic animals, 13th Edition. John Wiley & Sons, Ithaca and London.

Ettinger, S.J., Feldman, E.C., 2009. Textbook of veterinary internal medicine, 6th Edition. Elsevier Health Sciences, St. Louis, Missouri.

Eurell, J.A., F., B.L., 2013. Dellmann's Textbook of Veterinary Histology. Wiley.

Feldman, M., Friedman, L.S., Brandt, L.J., 2015. Sleisenger and Fordtran's gastrointestinal and liver disease: pathophysiology, diagnosis, management, 6th Edition. Elsevier Health Sciences, Philadelphia.

Fell, H.B., 1963. Studies of development in organ culture: summary, correlation, and speculation. Journal of the National Cancer Institute 11, 73-80.

Fellstrom, C., Gunnarsson, A., 1995. Phenotypical characterisation of intestinal spirochaetes isolated from pigs. Research in Veterinary Science 59, 1-4.

Fellstrom, C., Karlsson, M., Pettersson, B., Zimmerman, U., Gunnarsson, A., Aspan, A., 1999. Emended descriptions of indole negative and indole positive isolates of *Brachyspira* (*Serpulina*) *hyodysenteriae*. Veterinary Microbiology 70, 225-238.

Fenoglio, C.M., Kaye, G.I., Lane, N., 1973. Distribution of human colonic lymphatics in normal, hyperplastic, and adenomatous tissue. Its relationship to metastasis from small carcinomas in pedunculated adenomas, with two case reports. Gastroenterology 64, 51-66.

Field, M., Rao, M.C., Chang, E.B., 1989. Intestinal electrolyte transport and diarrheal disease (1). New England Journal of Medicine 321, 800-806.

Filipe, M.I., 1979. Mucins in the human gastrointestinal epithelium: a review. Investigative Cell Pathology 2, 195-216.

Fisher, L.F., Olander, H.J., 1981. Shedding of *Treponema hyodysenteriae*, transmission of disease, and agglutinin response to pigs convalescent from swine dysentery. American Journal of Veterinary Medicine 42, 450-455.

Fletcher, P.S., Elliott, J., Grivel, J.C., Margolis, L., Anton, P., McGowan, I., Shattock, R.J., 2006. Ex vivo culture of human colorectal tissue for the evaluation of candidate microbicides. AIDS 20, 1237-1245.

Forstner, G., 1995. Signal transduction, packaging and secretion of mucins. Annual Reviews of Physiology 57, 585-605.

Fraser, I.D., Condon, R.E., Schulte, W.J., DeCosse, J.J., Cowles, V.E., 1981. Longitudinal muscle of muscularis externa in human and nonhuman primate colon. Archives of Surgery 116, 61-63.

Fredricks, D.N., Relman, D.A., 1996. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. Clininical Microbiology Reviews 9, 18-33.

Fujisawa, T., Velichko, S., Thai, P., Hung, L.Y., Huang, F., Wu, R., 2009. Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. Journal of Immunology 183, 6236-6243.

Gawenis, L.R., Bradford, E.M., Prasad, V., Lorenz, J.N., Simpson, J.E., Clarke, L.L., Woo, A.L., Grisham, C., Sanford, L.P., Doetschman, T., Miller, M.L., Shull, G.E., 2007. Colonic anion secretory defects and metabolic acidosis in mice lacking the NBC1 Na+/HCO3- cotransporter. Journal of Biological Chemistry 282, 9042-9052.

Gebhart, C.J., Chander, Y., Vannucci, F., Kelley, M. 2012. Bacterial enteric diseases of growing pigs: dysentery and proliferative enteropathy. In International Pig Veterinary Society (Jeju, Korea), 48-50.

Geibel, J.P., Rajendran, V.M., Binder, H.J., 2001. Na(+)-dependent fluid absorption in intact perfused rat colonic crypts. Gastroenterology 120, 144-150.

Gihring, T.M., Green, S.J., Schadt, C.W., 2012. Massively parallel rRNA gene sequencing exacerbates the potential for biased community diversity comparisons due to variable library sizes. Environmental Microbiology 14, 285-290.

Girish, V., Vijayalakshmi, A., 2004. Affordable image analysis using NIH Image/ImageJ. Indian Journal of Cancer 41, 47.

Gitter, A.H., Bendfeldt, K., Schmitz, H., Schulzke, J.D., Bentzel, C.J., Fromm, M., 2000a. Epithelial barrier defects in HT-29/B6 colonic cell monolayers induced by tumor necrosis factor-alpha. Annals of the New York Academy of Sciences 915, 193-203.

Gitter, A.H., Bendfeldt, K., Schulzke, J.D., Fromm, M., 2000b. Trans/paracellular, surface/crypt, and epithelial/subepithelial resistances of mammalian colonic epithelia. Pflügers Archiv - European Journal of Physiology 439, 477-482.

Giuffra, E., Kijas, J.M., Amarger, V., Carlborg, O., Jeon, J.T., Andersson, L., 2000. The origin of the domestic pig: independent domestication and subsequent introgression. Genetics 154, 1785-1791.

Gledhill, A., Cole, F.M., 1984. Significance of basement membrane thickening in the human colon. Gut 25, 1085-1088.

Glock, R.D., 1971. Studies on the etiology, hematology and pathology of swine dysentery. Iowa State University, Ames.

Glock, R.D., Harris, D.L., Kluge, J.P., 1974. localization of spirochetes with the structural characteristics of *Treponema hyodysenteriae* in the lesions of swine dysentery. Infection and Immunity 9, 167-178.

Godl, K., Johansson, M.E., Lidell, M.E., Morgelin, M., Karlsson, H., Olson, F.J., Gum, J.R., Jr., Kim, Y.S., Hansson, G.C., 2002. The N terminus of the MUC2 mucin forms trimers that are held together within a trypsin-resistant core fragment. Journal of Biological Chemistry 277, 47248-47256.

Gordon, J.I., Hermiston, M.L., 1994. Differentiation and self-renewal in the mouse gastrointestinal epithelium. Current Opinions in Cell Biology 6, 795-803.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29, 644-652.

Greger, R., 2000. Role of CFTR in the colon. Annual Reviews of Physiology 62, 467-491.

Greger, R., Mall, M., Bleich, M., Ecke, D., Warth, R., Riedemann, N., Kunzelmann, K., 1996. Regulation of epithelial ion channels by the cystic fibrosis transmembrane conductance regulator. Journal of Molecular Medicine 74, 527-534.

Greger, R., Schreiber, R., Mall, M., Wissner, A., Hopf, A., Briel, M., Bleich, M., Warth, R., Kunzelmann, K., 2001. Cystic fibrosis and CFTR. Pflügers Archiv - European Journal of Physiology 443 Suppl 1, S3-7.

Guarino, A., Bisceglia, M., Canani, R.B., Boccia, M.C., Mallardo, G., Bruzzese, E., Massari, P., Rappuoli, R., Telford, J., 1998. Enterotoxic effect of the vacuolating toxin produced by *Helicobacter pylori* in Caco-2 cells. Journal of Infectious Diseases 178, 1373-1378.

Guarino, A., Canani, R.B., Pozio, E., Terracciano, L., Albano, F., Mazzeo, M., 1994. Enterotoxic effect of stool supernatant of *Cryptosporidium*-infected calves on human jejunum. Gastroenterology 106, 28-34.

Gum, J.R., Jr., Hicks, J.W., Toribara, N.W., Siddiki, B., Kim, Y.S., 1994. Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. Journal of Biological Chemistry 269, 2440-2446.

Halm, D.R., Halm, S.T., 1999. Secretagogue response of goblet cells and columnar cells in human colonic crypts. American Journal of Physiology 277, C501-522.

Halm, D.R., Halm, S.T., DiBona, D.R., Frizzell, R.A., Johnson, R.D., 1995. Selective stimulation of epithelial cells in colonic crypts: relation to active chloride secretion. American Journal of Physiology 269, C929-942.

Hampson, D.J., Combs, B.G., Harders, S.J., Connaughton, I.D., Fahy, V.A., 1991. Isolation of *Treponema hyodysenteriae* from a wild rat living on a piggery. Autralian Veterinary Journal 68, 308.

Hampson, D.J., Fellstrom, C., Thomson, J.R. 2006. Swine Dysentery, In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.) Diseases of Swine. Blackwell Publishing, Ames, IO, 785-805.

Hampson, D.J., Robertson, I.D., La, T., Oxberry, S.L., Pethick, D.W., 2000. Influences of diet and vaccination on colonisation of pigs by the intestinal spirochaete *Brachyspira* (*Serpulina*) *pilosicoli*. Veterinary Microbiology 73, 75-84.

Hansen, C.F., Phillips, N.D., La, T., Hernandez, A., Mansfield, J., Kim, J.C., Mullan, B.P., Hampson, D.J., Pluske, J.R., 2010. Diets containing inulin but not lupins help to prevent swine dysentery in experimentally challenged pigs. Journal of Animal Science 88, 3327-3336.

Hansson, G.C., Johansson, M.E., 2010. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Gut Microbes 1, 51-54.

Haque, A., Bowe, F., Fitzhenry, R.J., Frankel, G., Thomson, M., Heuschkel, R., Murch, S., Stevens, M.P., Wallis, T.S., Phillips, A.D., Dougan, G., 2004. Early interactions of *Salmonella enterica* serovar typhimurium with human small intestinal epithelial explants. Gut 53, 1424-1430.

Harding, J.C., Baker, C.D., Tumber, A., McIntosh, K.A., Parker, S.E., Middleton, D.M., Hill, J.E., Ellis, J.A., Krakowka, S., 2008. Porcine circovirus-2 DNA concentration distinguishes wasting from nonwasting pigs and is correlated with lesion distribution, severity, and nucleocapsid staining intensity. Journal of Veterinary Diagnostic Investigation 20, 274-282.

Harding, J.C., Fernando, C., Costa, M.C., Patterson, A.H., Rubin, J.E., Hill, J.E. 2013. What have we learned about Swine Dysentery in Canada? In: Banff Pork Seminar, Banff, Canada, 2013, 175-178.

Harding, J.C.S., Chirino-Trejo, M., Fernando, C., Jacobson, M., Forster, Z., Hill, J.E. 2010a. Detection of a novel *Brachyspira* species associated with haemorrhagic and necrotizing colitis. In: International Pig Veterinary Society Congress, Vancouver, Canada, July 18-21, 2010, 740.

Harding, J.C.S., Chirino-Trejo, M., Vermette, C., Fernando, C., Jacobson, M., Forster, Z., Hill, J.E. 2010b. Detection of a novel *Brachyspira* species associated with haemorrhagic and necrotizing colitis. In: Proceedings of the Annual Western Canadian Association of Swine Veterinarians Conference, Saskatoon, SK, October 15-16, 65-70.

Harding, J.C.S., Hill, J.E., Chirino-Trejo, M., O'Connor, B., Rubin, J.E., Fernando, C. 2011. Detection of a novel *Brachyspira* species associated with haemorrhagic and necrotizing colitis. In Carlos Pijoan Symposium on Swine Dysentery (St. Paul, MN), 27-32.

Harris, D., Glock, R., 1971. Swine dysentery: a review. Iowa State University Veterinarian 33, 1.

Harris, D.L., Alexander, T.J., Whipp, S.C., Robinson, I.M., Glock, R.D., Matthews, P.J., 1978. Swine dysentery: studies of gnotobiotic pigs inoculated with *Treponema hyodysenteriae, Bacteroides vulgatus,* and *Fusobacterium necrophorum*. Journal of the Veterinary Medical Association 172, 468-471.

Harris, D.L., Glock, R.D., Christensen, C.R., Kinyon, J.M., 1972. Inoculation of pigs with *Treponema hyodysenteriae* (new species) and reproduction of the disease. Veterinary Medicine - Small Animal Clinic 67, 61-64.

Hasegawa, H., Skach, W., Baker, O., Calayag, M.C., Lingappa, V., Verkman, A.S., 1992. A multifunctional aqueous channel formed by CFTR. Science 258, 1477-1479.

Heazlewood, C.K., Cook, M.C., Eri, R., Price, G.R., Tauro, S.B., Taupin, D., Thornton, D.J., Png, C.W., Crockford, T.L., Cornall, R.J., Adams, R., Kato, M., Nelms, K.A., Hong, N.A., Florin, T.H., Goodnow, C.C., McGuckin, M.A., 2008. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med 5, e54.

Henle, F.G.J., 1837. *Symbolae ad anatomiam villorum intestinalium, imprimis eorum epithelii et vasorum lacteorum*.

Hidalgo, A., Carvajal, A., Vester, B., Pringle, M., Naharro, G., Rubio, P., 2011. Trends towards Lower Antimicrobial Susceptibility and Characterization of Acquired Resistance among Clinical Isolates of Brachyspira hyodysenteriae in Spain. Antimicrobial Agents and Chemotherapy 55, 3330-3337.

Hill, J.E., Hemmingsen, S.M., Goldade, B.G., Dumonceaux, T.J., Klassen, J., Zijlstra, R.T., Goh, S.H., Van Kessel, A.G., 2005. Comparison of ileum microflora of pigs fed corn-, wheat-, or barley-based diets by chaperonin-60 sequencing and quantitative PCR. Applied Environmental Microbiology 71, 867-875.

Hill, J.E., Penny, S.L., Crowell, K.G., Goh, S.H., Hemmingsen, S.M., 2004. cpnDB: a chaperonin sequence database. Genome Research 14, 1669-1675.

Hogan, P.G., Hapel, A.J., Doe, W.F., 1985. Lymphokine-activated and natural killer cell activity in human intestinal mucosa. Journal of Immunology 135, 1731-1738.

Hollander, D., 2002. Crohn's disease, TNF-alpha, and the leaky gut. The chicken or the egg? American Journal of Gastroenterology 97, 1867-1868.

Holt, S.C., 1978. Anatomy and chemistry of spirochetes. Microbiological Review 42, 114-160.

Hommez, J., Castryck, F., Haesebrouck, F., Devriese, L.A., 1998. Identification of porcine *Serpulina* strains in routine diagnostic bacteriology. Veterinary Microbiology 62, 163-169.

Hsu, T., Hutto, D.L., Minion, F.C., Zuerner, R.L., Wannemuehler, M.J., 2001. Cloning of a beta-hemolysin gene of *Brachyspira* (*Serpulina*) *hyodysenteriae* and its expression in *Escherichia coli*. Infection and Immunity 69, 706-711.

Hyatt, D.R., ter Huurne, A.A., van der Zeijst, B.A., Joens, L.A., 1994. Reduced virulence of *Serpulina hyodysenteriae* hemolysin-negative mutants in pigs and their potential to protect pigs against challenge with a virulent strain. Infection and Immunity 62, 2244-2248.

Ikuma, M., Kashgarian, M., Binder, H.J., Rajendran, V.M., 1999. Differential regulation of NHE isoforms by sodium depletion in proximal and distal segments of rat colon. American Journal of Physiology 276, G539-549.

Jacobson, M., Andersson, M., Lindberg, R., Fossum, C., Jensen-Waern, M., 2011. Microarray and cytokine analyses of field cases of pigs with diarrhoea. Veterinary Microbiology 153, 307-314.

Jacobson, M., Fellstrom, C., Lindberg, R., Wallgren, P., Jensen-Waern, M., 2004. Experimental swine dysentery: comparison between infection models. Journal of Medical Microbiology 53, 273-280.

Jansson, D.S., Johansson, K.E., Olofsson, T., Rasback, T., Vagsholm, I., Pettersson, B., Gunnarsson, A., Fellstrom, C., 2004. *Brachyspira hyodysenteriae* and other strongly beta-haemolytic and indole-positive spirochaetes isolated from mallards (*Anas platyrhynchos*). Journal of Medical Microbiology 53, 293-300.

Jarry, A., Merlin, D., Hopfer, U., Laboisse, C.L., 1994a. Cyclic AMP-induced mucin exocytosis is independent of Cl- movements in human colonic epithelial cells (HT29-Cl.16E). Journal of Biochemistry 304 (Pt 3), 675-678.

Jarry, A., Merlin, D., Velcich, A., Hopfer, U., Augenlicht, L.H., Laboisse, C.L., 1994b. Interferon-gamma modulates cAMP-induced mucin exocytosis without affecting mucin gene expression in a human colonic goblet cell line. European Journal of Pharmacology 267, 95-103.

Jenkinson, S.R., Wingar, C.R., 1981. Selective medium for the isolation of *Treponema hyodysenteriae*. Veterinary Records 109, 384-385.

Jensen, N.S., Stanton, T.B., Swayne, D.E., 1996. Identification of the swine pathogen *Serpulina hyodysenteriae* in rheas (*Rhea americana*). Veterinary Microbiology 52, 259-269.

Jensen, T.K., Boye, M., Moller, K., Leser, T.D., Jorsal, S.E., 1998. Association of *Serpulina hyodysenteriae* with the colonic mucosa in experimental swine dysentery studied by fluorescent *in situ* hybridization. APMIS 106, 1061-1068.

Jensen, T.K., Christensen, A.S., Boye, M., 2010. *Brachyspira murdochii* colitis in pigs. Veterinary Pathology Online 47, 334-338.

Jensen, T.K., Christensen, B.B., Boye, M., 2006. *Lawsonia intracellularis* infection in the large intestines of pigs. APMIS 114, 255-264.

Jenssen, H., Hamill, P., Hancock, R.E., 2006. Peptide antimicrobial agents. Clinical Microbiology Reviews 19, 491-511.

Joens, L.A., Songer, J.G., Harris, D.L., Kinyon, J.M., 1979. Comparison of selective culture and serologic agglutination of Treponema hyodysenteriae for diagnosis of swine dysentery. Veterinary Records 105, 463-465.

Johansson, M.E., Gustafsson, J.K., Holmen-Larsson, J., Jabbar, K.S., Xia, L., Xu, H., Ghishan, F.K., Carvalho, F.A., Gewirtz, A.T., Sjovall, H., Hansson, G.C., 2014. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. Gut 63, 281-291.

Johansson, M.E., Larsson, J.M., Hansson, G.C., 2011. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proceedings of the National Academy of Sciences 108 Suppl 1, 4659-4665.

Johansson, M.E., Phillipson, M., Petersson, J., Velcich, A., Holm, L., Hansson, G.C., 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proceedings of the National Academy of Sciences 105, 15064-15069.

Johansson, M.E., Thomsson, K.A., Hansson, G.C., 2009. Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. Journal of Proteome Research 8, 3549-3557.

Jonasson, R., Johannisson, A., Jacobson, M., Fellstrom, C., Jensen-Waern, M., 2004. Differences in lymphocyte subpopulations and cell counts before and after experimentally induced swine dysentery. Journal of Medical Microbiology 53, 267-272.

Jones, G.F., Ward, G.E., Murtaugh, M.P., Lin, G., Gebhart, C.J., 1993. Enhanced detection of intracellular organism of swine proliferative enteritis, ileal symbiont intracellularis, in feces by polymerase chain reaction. Journal of Clinical Microbiology 31, 2611-2615.

Junqueira, L.C., Carneiro, J., 2005. Basic histology text and atlas. London: McGraw Hill, 2005.

Karam, S.M., 1999. Lineage commitment and maturation of epithelial cells in the gut. Frontiers in Bioscience 4, D286-298.

Kennedy, M.J., Yancey, R.J., Jr., 1996. Motility and chemotaxis in *Serpulina hyodysenteriae*. Veterinary Microbiology 49, 21-30.

Kesisoglou, F., Schmiedlin-Ren, P., Fleisher, D., Roessler, B., Zimmermann, E.M., 2006. Restituting intestinal epithelial cells exhibit increased transducibility by adenoviral vectors. Journal of Gene Medicine 8, 1379-1392.

Kik, M.J., Koninkx, J.F., van den Muysenberg, A., Hendriksen, F., 1991. Pathological effects of *Phaseolus vulgaris* isolectins on pig jejunal mucosa in organ culture. Gut 32, 886-892.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J., Isaacson, R.E., 2011. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. Veterinary Microbiology 153, 124-133.

Kim, Y.S., Ho, S.B., 2010. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Current Gastroenterology Reports 12, 319-330.

Kinyon, J.M., Harris, D.L., Glock, R.D., 1977. Enteropathogenicity of various isolates of *Treponema hyodysenteriae*. Infection and Immunity 15, 638-646.

Klein, B.G., 2013. Cunningham's textbook of veterinary physiology, 2nd Edition. Saunders Elsevier, Missouri.

Koch, R., 1876. Untersuchungen ueber Bakterien V. Die Aetiologie der Milzbrand-Krankheit, begruendent auf die Entwicklungsgeschichte des Bacillus Anthracis. Beitr. z. Biol. D. Pflanzen 2: 277-310. Milestones in Microbiology 1556.

Kockerling, A., Fromm, M., 1993. Origin of cAMP-dependent Cl- secretion from both crypts and surface epithelia of rat intestine. American Journal of Physiology 264, C1294-1301.

Koh, H.W., Kim, M.S., Lee, J.S., Kim, H., Park, S.J., 2015. Changes in the swine gut microbiota in response to porcine epidemic diarrhea infection. Microbes and Environments 30, 284-287.

Komarek, V., Maderner, A., Spergser, J., Weissenbock, H., 2009. Infections with weakly haemolytic *Brachyspira* species in pigs with miscellaneous chronic diseases. Veterinary Microbiology 134, 311-317.

Konstantinov, S.R., Favier, C.F., Zhu, W.Y., Williams, B.A., Kluss, J., Souffrant, W.B., de Vos, W.M., Akkermans, A.D.L., Smidt, H., 2004. Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. Animal Research 53, 317-324.

Koyama, Y., Yamamoto, T., Tani, T., Nihei, K., Kondo, D., Funaki, H., Yaoita, E., Kawasaki, K., Sato, N., Hatakeyama, K., Kihara, I., 1999. Expression and localization of aquaporins in rat gastrointestinal tract. American Journal of Physiology 276, C621-627.

Kruse, R., Essen-Gustavsson, B., Fossum, C., Jensen-Waern, M., 2008. Blood concentrations of the cytokines IL-1beta, IL-6, IL-10, TNF-alpha and IFN-gamma during experimentally induced swine dysentery. Acta Veterinaria Scandinavica 50, 32.

Kunkle, R.A., Kinyon, J.M., 1988. Improved selective medium for the isolation of *Treponema hyodysenteriae*. Journal of Clinical Microbiology 26, 2357-2360.

La, T., Collins, A.M., Phillips, N.D., Oksa, A., Hampson, D.J., 2006. Development of a multiplex-PCR for rapid detection of the enteric pathogens *Lawsonia intracellularis, Brachyspira hyodysenteriae,* and *Brachyspira pilosicoli* in porcine faeces. Letters in Applied Microbiology 42, 284-288.

La, T., Hampson, D.J., 2001. Serologic detection of *Brachyspira* (*Serpulina*) *hyodysenteriae* infections. Animal Health Research Reviews 2, 45-52.

La, T., Phillips, N.D., Hampson, D.J., 2003. Development of a duplex PCR assay for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in pig feces. Journal of Clinical Microbiology 41, 3372-3375.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9, 357-359.

Lawson, G.H., Mackie, R.A., Smith, D.G., McOrist, S., 1995. Infection of cultured rat enterocytes by Ileal symbiont intracellularis depends on host cell function and actin polymerisation. Veterinary Microbiology 45, 339-350.

Lee, D.H., Zo, Y.G., Kim, S.J., 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Journal of Applied Environmental Microbiology 62, 3112-3120.

Leite, M.F., Hirata, K., Pusl, T., Burgstahler, A.D., Okazaki, K., Ortega, J.M., Goes, A.M., Prado, M.A., Spray, D.C., Nathanson, M.H., 2002. Molecular basis for pacemaker cells in epithelia. Journal of Biological Chemistry 277, 16313-16323.

Lembo-Fazio, L., Nigro, G., Noel, G., Rossi, G., Chiara, F., Tsilingiri, K., Rescigno, M., Rasola, A., Bernardini, M.L., 2011. Gadd45alpha activity is the principal effector of *Shigella* mitochondria-dependent epithelial cell death *in vitro* and *ex vivo*. Cell Death and Disease 2, e122.

Leser, T.D., Lindecrona, R.H., Jensen, T.K., Jensen, B.B., Møller, K., 2000. Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with *Brachyspira hyodysenteriae*. Applied and Environmental Microbiology 66, 3290-3296.

Levine, D.S., Haggitt, R.C., 1989. Normal histology of the colon. American Journal of Surgical Pathology 13, 966-984.

Levine, N.D., Peterson, E.H., Graham, R., 1945. Studies on swine enteritis: *Salmonella* and other enteric organisms isolated from diseased and normal swine. American Journal of Veterinary Research 6, 242-246.

Levy, J., 2000. The effects of antibiotic use on gastrointestinal function. American Journal of Gastroenterology 95, S8-10.

Lieberkühn, J.N., 1745. *Dissertatio anatomico-physiologica de fabrica et actione villorum intestinorum tenuium hominis. Iconibus aeri incisis illustrata*. University of Madrid, Madrid.

Links, M.G., Chaban, B., Hemmingsen, S.M., Muirhead, K., Hill, J.E., 2013. mPUMA: a computational approach to microbiota analysis by de novo assembly of operational taxonomic units based on protein-coding barcode sequences. Microbiome 1, 23.

Lipkin, M., Deschner, E., 1968. Comparative analysis of cell renewal in the gastrointestinal tract of newborn hamster. Experimental Cell Research 49, 1-12.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Loo, D.D., Kaunitz, J.D., 1989. Ca2+ and cAMP activate K+ channels in the basolateral membrane of crypt cells isolated from rabbit distal colon. Journal of Membrane Biology 110, 19-28.

Lupp, C., Robertson, M.L., Wickham, M.E., Sekirov, I., Champion, O.L., Gaynor, E.C., Finlay, B.B., 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe 2, 204.

Lytle, C., Forbush, B., 3rd, 1996. Regulatory phosphorylation of the secretory Na-K-Cl cotransporter: modulation by cytoplasmic Cl. American Journal of Physiology 270, C437-448.

Ma, T.Y., Anderson, J.M. 2006. Chapter 61 - Tight Junctions and the Intestinal Barrier, In: Wood, L.R., Johnson, Kim E., Barret, Fayez K., Gishan, Juanita L., Merchant, Hamid M., Said, Jackie D. (Ed.) Physiology of the Gastrointestinal Tract (Fourth Edition). Academic Press, Burlington, 1559-1594.

Ma, T.Y., Iwamoto, G.K., Hoa, N.T., Akotia, V., Pedram, A., Boivin, M.A., Said, H.M., 2004. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. American Journal of Gastroenterology 286, G367-376.

Madara, J.L., Stafford, J., 1989. Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. Journal of Clinical Investigation 83, 724-727.

Mahu, M., de Jong, E., De Pauw, N., Vande Maele, L., Vandenbroucke, V., Vandersmissen, T., Miry, C., Pasmans, F., Haesebrouck, F., Martel, A., Boyen, F., 2014. First isolation of "*Brachyspira hampsonii*" from pigs in Europe. Veterinary Records 174, 47.

Mappley, L.J., Tchorzewska, M.A., Cooley, W.A., Woodward, M.J., La Ragione, R.M., 2011. Lactobacilli antagonize the growth, motility, and adherence of *Brachyspira pilosicoli*: a potential intervention against avian intestinal spirochetosis. Applied Environmental Microbiology 77, 5402-5411.

Marshall, T., Allen, A., 1978. The isolation and characterization of the high-molecular-weight glycoprotein from pig colonic mucus. Journal of Biochemistry 173, 569-578.

Martinez-Lobo, F.J., Hidalgo, A., Garcia, M., Arguello, H., Naharro, G., Carvajal, A., Rubio, P., 2013. First identification of "*Brachyspira hampsonii*" in wild european waterfowl. PLoS One 8, e82626.

Matsuo, K., Ota, H., Akamatsu, T., Sugiyama, A., Katsuyama, T., 1997. Histochemistry of the surface mucous gel layer of the human colon. Gut 40, 782-789.

McCluskey, J., Hannigan, J., Harris, J.D., Wren, B., Smith, D.G., 2002. LsaA, an antigen involved in cell attachment and invasion, is expressed by *Lawsonia intracellularis* during infection *in vitro* and *in vivo*. Infection and Immunity 70, 2899-2907.

McCormick, B.A., Hofman, P.M., Kim, J., Carnes, D.K., Miller, S.I., Madara, J.L., 1995. Surface attachment of *Salmonella typhimurium* to intestinal epithelia imprints the subepithelial matrix with gradients chemotactic for neutrophils. Journal of Cell Biology 131, 1599-1608.

McOrist, S., Gebhart, C.J., Boid, R., Barns, S.M., 1995a. Characterization of *Lawsonia intracellularis* gen. nov., sp. nov., the obligately intracellular bacterium of porcine proliferative enteropathy. International Journal of Systematic Bacteriology 45, 820-825.

McOrist, S., Jasni, S., Mackie, R.A., Berschneider, H.M., Rowland, A.C., Lawson, G.H., 1995b. Entry of the bacterium ileal symbiont intracellularis into cultured enterocytes and its subsequent release. Research in Veterinary Science 59, 255-260.

Meyer, R., Simon, J., Byerly, C., 1975. The etiology of swine dysentery III. The role of selected gram-negative obligate anaerobes. Veterinary Pathology Online 12, 46-54.

Meyer, R.C., Simon, J., Byerly, C.S., 1974a. The etiology of swine dysentery. I. Oral inoculation of germ-free swine with *Treponema hyodysenteriae* and *Vibrio coli*. Veterinary Pathology Online 11, 515-526.

Meyer, R.C., Simon, J., Byerly, C.S., 1974b. The etiology of swine dysentery. II. Effect of a known microbial flora, weaning and diet on disease production in gnotobiotic and conventional swine. Veterinary Pathology Online 11, 527-534.

Meyerholz, D.K., Stabel, T.J., 2003. Comparison of early ileal invasion by *Salmonella enterica* serovars Choleraesuis and Typhimurium. Veterinary Pathology Online 40, 371-375.

Milner, J.A., Sellwood, R., 1994. Chemotactic response to mucin by *Serpulina hyodysenteriae* and other porcine spirochetes: potential role in intestinal colonization. Infection and Immunity 62, 4095-4099.

Mitchell, J.D., Mitchell, J., Peters, T.J., 1974. Enzyme changes in human small bowel mucosa during culture in vitro. Gut 15, 805-811.

Montagne, L., Piel, C., Lalles, J.P., 2004. Effect of diet on mucin kinetics and composition: nutrition and health implications. Nutrition Reviews 62, 105-114.

Morse, W.F., 1908. The collection and disposal of municipal waste. Municipal Journal and Engineer.

Moxley, R.A., Duhamel, G.E., 1999. Comparative pathology of bacterial enteric diseases of swine. Advances in Experimental Medicine and Biology 473, 83-101.

Muniappa, N., Ramanathan, M.R., Tarara, R., Westerman, R., Mathiesen, M., Duhamel, G., 1998. Attachment of human and rhesus *Serpulina pilosicoli* to cultured cells and comparison with a chick infection model. Journal Of Spirochetal And Tick Borne Diseases 5, 44-53.

Naresh, R., Song, Y., Hampson, D.J., 2009. The intestinal spirochete *Brachyspira pilosicoli* attaches to cultured Caco-2 cells and induces pathological changes. PLoS One 4, e8352.

Nathues, H., Oliveira, C.J., Wurm, M., Grosse Beilage, E., Givisiez, P.E., 2007. Simultaneous detection of *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli* and *Lawsonia intracellularis* in porcine faeces and tissue samples by multiplex-PCR. Journal of Veterinary Medicine. A- Physiology, pathology, clinical medicine 54, 532-538.

Neef, N.A., Lysons, R.J., Trott, D.J., Hampson, D.J., Jones, P.W., Morgan, J.H., 1994. Pathogenicity of porcine intestinal spirochetes in gnotobiotic pigs. Infection and Immunity 62, 2395-2403.

Neutra, M.R., O'Malley, L.J., Specian, R.D., 1982. Regulation of intestinal goblet cell secretion. II. A survey of potential secretagogues. American Journal of Physiology 242, G380-387.

Nibbelink, S.K., Wannemuehler, M.J., 1991. Susceptibility of inbred mouse strains to infection with *Serpula* (*Treponema*) *hyodysenteriae*. Infection and Immunity 59, 3111-3118.

Norris, F.A., Wilson, M.P., Wallis, T.S., Galyov, E.E., Majerus, P.W., 1998. SopB, a protein required for virulence of *Salmonella dublin*, is an inositol phosphate phosphatase. Proceedings of the National Academy of Sciences 95, 14057-14059.

Novotna, M., Skardova, O., 2002. *Brachyspira hyodysenteriae*: detection, identification and antibiotic susceptibility. Veterinarni Medicina 47, 104-109.

Onuma, H., Mastui, C., Morohashi, M., 2001. Quantitative analysis of the proliferation of epidermal cells using a human skin organ culture system and the effect of DbcAMP using markers of proliferation (BrdU, Ki-67, PCNA). Archives of Dermatological Research 293, 133-138.

Osorio, J., Carvajal, A., Naharro, G., Rubio, P., La, T., Phillips, N.D., Hampson, D.J., 2013. Identification of weakly haemolytic *Brachyspira* isolates recovered from pigs with diarrhoea in Spain and Portugal and comparison with results from other countries. Research in Veterinary Science 95, 861-869.

Palzer, A., Ritzmann, M., Poschinger, K., Staßen, T., Nathues, H., Heinritzi, K., 2008. Der Klinische Fall-Frage und Antwort: Enteritis bei Mastschweinen mit Nachweis von Brachyspira murdochii. Tierärztliche Praxis Großtiere 36, 257-265.

Paradis, M.A., Gottschalk, M., Rajic, A., Ravel, A., Wilson, J.B., Aramini, J., McClure, C.A., Dick, C.P., 2007. Seroprevalence of *Lawsonia intracellularis* in different swine populations in 3 provinces in Canada. Canadian Veterinary Journal 48, 57-62.

Paszti-Gere, E., Csibrik-Nemeth, E., Szeker, K., Csizinszky, R., Jakab, C., Galfi, P., 2012. Acute oxidative stress affects IL-8 and TNF-alpha expression in IPEC-J2 porcine epithelial cells. Inflammation 35, 994-1004.

Patterson, A.H., Rubin, J.E., Fernando, C., Costa, M.O., Harding, J.C., Hill, J.E., 2013. Fecal shedding of *Brachyspira* spp. on a farrow-to-finish swine farm with a clinical history of "*Brachyspira hampsonii*"-associated colitis. BMC Veterinary Research 9, 137.

Pearce, G.P., 1999. Epidemiology of enteric disease in grower-finisher pigs: a postal survey of pig producers in England. Veterinary Record 144, 338-342.

Pernodet, N., Maaloum, M., Tinland, B., 1997. Pore size of agarose gels by atomic force microscopy. Electrophoresis 18, 55-58.

Petersen, H.H., Nielsen, E.O., Hassing, A.G., Ersboll, A.K., Nielsen, J.P., 2008. Prevalence of clinical signs of disease in Danish finisher pigs. Veterinary Records 162, 377-382.

Peterson, J.W., Ochoa, L.G., 1989. Role of prostaglandins and cAMP in the secretory effects of cholera toxin. Science 245, 857-859.

Pettersson, B., Fellstrom, C., Andersson, A., Uhlen, M., Gunnarsson, A., Johansson, K.E., 1996. The phylogeny of intestinal porcine spirochetes (*Serpulina* species) based on sequence analysis of the 16S rRNA gene. Journal of Bacteriology 178, 4189-4199.

Phillips, N.D., La, T., Amin, M.M., Hampson, D.J., 2010. *Brachyspira intermedia* strain diversity and relationships to the other indole-positive *Brachyspira* species. Veterinary Microbiology 143, 246-254.

Pie, S., Lalles, J.P., Blazy, F., Laffitte, J., Seve, B., Oswald, I.P., 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. Journal of Nutrition 134, 641-647.

Pierce, N.F., 1973. Differential inhibitory effects of cholera toxoids and ganglioside on the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Journal of Experimental Medicine 137, 1009-1023.

Plebani, M., Carraro, P., 1997. Mistakes in a stat laboratory: types and frequency. Clinical Chemistry 43, 1348-1351.

Pluske, J.R., Siba, P.M., Pethick, D.W., Durmic, Z., Mullan, B.P., Hampson, D.J., 1996. The incidence of swine dysentery in pigs can be reduced by feeding diets that limit the amount of fermentable substrate entering the large intestine. Journal of Nutrition 126, 2920-2933.

Podolsky, D.K., Isselbacher, K.J., 1983. Composition of human colonic mucin. Selective alteration in inflammatory bowel disease. Journal of Clinical Investigation 72, 142-153.

Powell, D.W., 1981. Barrier function of epithelia. American Journal of Physiology 241, G275-288.

Quinlan, J.M., Yu, W.Y., Hornsey, M.A., Tosh, D., Slack, J.M.W., 2006. *In vitro* culture of embryonic mouse intestinal epithelium: cell differentiation and introduction of reporter genes. BMC Developmental Biology 6.

Rajendran, V.M., Black, J., Ardito, T.A., Sangan, P., Alper, S.L., Schweinfest, C., Kashgarian, M., Binder, H.J., 2000. Regulation of DRA and AE1 in rat colon by dietary Na depletion. American Journal of Gastroenterology 279, G931-942.

Rajendran, V.M., Geibel, J., Binder, H.J., 1999. Role of Cl channels in Cl-dependent Na/H exchange. American Journal of Physiology 276, G73-78.

Råsbäck, T., Fellstrom, C., Bergsjo, B., Cizek, A., Collin, K., Gunnarsson, A., Jensen, S.M., Mars, A., Thomson, J., Vyt, P., Pringle, M., 2005. Assessment of diagnostics and antimicrobial susceptibility testing of *Brachyspira* species using a ring test. Veterinary Microbiology 109, 229-243.

Råsbäck, T., Jansson, D.S., Johansson, K.-E., Fellström, C., 2007a. A novel enteropathogenic, strongly haemolytic spirochaete isolated from pig and mallard, provisionally designated '*Brachyspira suanatina*' sp. nov. Environmental Microbiology 9, 983-991.

Rasback, T., Johansson, K.E., Jansson, D.S., Fellstrom, C., Alikhani, M.Y., La, T., Dunn, D.S., Hampson, D.J., 2007. Development of a multilocus sequence typing scheme for intestinal spirochaetes within the genus *Brachyspira*. Microbiology 153, 4074-4087.

Råsbäck, T., Johansson, K.E., Jansson, D.S., Fellström, C., Alikhani, M.Y., La, T., Dunn, D.S., Hampson, D.J., 2007b. Development of a multilocus sequence typing scheme for intestinal spirochaetes within the genus *Brachyspira*. Microbiology 153, 4074-4087.

Read, N.W., Krejs, G.J., Read, M.G., Santa Ana, C.A., Morawski, S.G., Fordtran, J.S., 1980. Chronic diarrhea of unknown origin. Gastroenterology 78, 264-271.

Reed, W.M., Olander, H.J., Thacker, H.L., 1986. Studies on the pathogenesis of *Salmonella typhimurium* and *Salmonella choleraesuis* var kunzendorf infection in weanling pigs. American Journal of Veterinary Research 47, 75-83.

Reiss, B., Williams, G.M., 1979. Conditions affecting prolonged maintenance of mouse and rat colon in organ culture. In Vitro 15, 877-890.

Rerat, A., Fiszlewicz, M., Giusi, A., Vaugelade, P., 1987. Influence of meal frequency on postprandial variations in the production and absorption of volatile fatty acids in the digestive tract of conscious pigs. Journal of Animal Science 64, 448-456.

Resau, J.H., Sakamoto, K., Cottrell, J.R., Hudson, E.A., Meltzer, S.J., 1991. Explant Organ-Culture - a Review. Cytotechnology 7, 137-149.

Riegler, M., Lotz, M., Sears, C., Pothoulakis, C., Castagliuolo, I., Wang, C.C., Sedivy, R., Sogukoglu, T., Cosentini, E., Bischof, G., Feil, W., Teleky, B., Hamilton, G., LaMont, J.T., Wenzl, E., 1999. *Bacteroides fragilis* toxin 2 damages human colonic mucosa *in vitro*. Gut 44, 504-510.

Rifaat, M.K., Iseri, O.A., Gottlieb, L.S., 1983. An ultrastructural study of the “extraneous coat” of human colonic mucosa. Gastroenterology 48, 593-601.

Robert, M.E., Singh, S.K., Ikuma, M., Jain, D., Ardito, T., Binder, H.J., 2001. Morphology of isolated colonic crypts. Cells Tissues Organs 168, 246-251.

Robinson, I.M., Whipp, S.C., Bucklin, J.A., Allison, M.J., 1984. Characterization of predominant bacteria from the colons of normal and dysenteric pigs. Applied and Environmental Microbiology 48, 964-969.

Rohde, J., Habighorst-Blome, K., Seehusen, F., 2014. "*Brachyspira hampsonii*" clade I isolated from Belgian pigs imported to Germany. Veterinary Microbiology 168, 432-435.

Rohde, J., Rothkamp, A., Gerlach, G.F., 2002. Differentiation of porcine *Brachyspira* species by a novel nox pcr-based restriction fragment length polymorphism analysis. Journal of Clinical Microbiology 40, 2598-2600.

Rosey, E.L., Kennedy, M.J., Yancey, R.J., Jr., 1996. Dual *fla*A1 *fla*B1 mutant of *Serpulina hyodysenteriae* expressing periplasmic flagella is severely attenuated in a murine model of swine dysentery. Infection and Immunity 64, 4154-4162.

Round, J.L., Mazmanian, S.K., 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proceedings of the National Academy of Sciences 107, 12204-12209.

Round, J.L., O'Connell, R.M., Mazmanian, S.K., 2010. Coordination of tolerogenic immune responses by the commensal microbiota. Journal of Autoimmunity 34, J220-225.

Rubin, J.E., Costa, M.O., Hill, J.E., Kittrell, H.E., Fernando, C., Huang, Y., O'Connor, B., Harding, J.C.S., 2013a. Reproduction of mucohaemorrhagic diarrhea and colitis indistinguishable from Swine Dysentery following experimental inoculation with "*Brachyspira hampsonii*" strain 30446. PLoS One 8, e57146.

Rubin, J.E., Harms, N.J., Fernando, C., Soos, C., Detmer, S.E., Harding, J.C., Hill, J.E., 2013b. Isolation and characterization of *Brachyspira* spp. including "*Brachyspira hampsonii*" from lesser snow geese (*Chen caerulescens caerulescens*) in the Canadian Arctic. Microbial Ecology 66, 813-822.

Said, H.M., Seetharam, B. 2006. Chapter 71 - Intestinal Absorption of Water-Soluble Vitamins, In: Wood, L.R., Johnson, Kim E., Barret, Fayez K., Gishan, Juanita L., Merchant, Hamid M., Said, Jackie D. (Ed.) Physiology of the Gastrointestinal Tract. Academic Press, Burlington, 1791-1825.

Saunders, D.R., Sillery, J., Rachmilewitz, D., 1975. Effect of dioctyl sodium sulfosuccinate on structure and function of rodent and human intestine. Gastroenterology 69, 380-386.

Schauser, K., Olsen, J.E., Larsson, L.I., 2004. Immunocytochemical studies of *Salmonella* Typhimurium invasion of porcine jejunal epithelial cells. Journal of Medical Microbiology 53, 691-695.

Schauser, K., Olsen, J.E., Larsson, L.I., 2005. *Salmonella* Typhimurium infection in the porcine intestine: evidence for caspase-3-dependent and -independent programmed cell death. Histochemistry and Cell Biology 123, 43-50.

Schellenberg, J., Links, M.G., Hill, J.E., Hemmingsen, S.M., Peters, G.A., Dumonceaux, T.J. 2011. Pyrosequencing of chaperonin-60 (*cpn*60) amplicons as a means of determining microbial community composition In: Kwon, Y.M., Ricke, S.C. (Eds.) Methods in Molecular Biology. Humana Press, 143-158.

Schiff, L.J., 1975. Organ cultures of rat and hamster colon. In Vitro 11, 46-49.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75, 7537-7541.

Schmall, L.M., Argenzio, R.A., Whipp, S.C., 1983. Pathophysiologic features of swine dysentery: cyclic nucleotide-independent production of diarrhea. American Journal of Veterinary Research 44, 1309-1316.

Schonhoff, S.E., Giel-Moloney, M., Leiter, A.B., 2004. Minireview: Development and differentiation of gut endocrine cells. Endocrinology 145, 2639-2644.

Schüller, S., Frankel, G., Phillips, A.D., 2004. Interaction of Shiga toxin from *Escherichia coli* with human intestinal epithelial cell lines and explants: Stx2 induces epithelial damage in organ culture. Cellular microbiology 6, 289-301.

Schwartz, K. 1999. Salmonellosis, In: Press, I.S.U. (Ed.) Diseases of Swine.Iowa, 535-551.

Schwartz, K. 2011. *Brachyspira*: What's Happening in Iowa and Why. In Western Canadian Association of Swine Veterinarians Conference, 25-33.

Sekirov, I., Finlay, B.B., 2009. The role of the intestinal microbiota in enteric infection. The Journal of Physiology 587, 4159-4167.

Shamsuddin, A.K., Barrett, L.A., Autrup, H., Harris, C.C., Trump, B.F., 1978. Long-term organ culture of adult rat colon. Pathology - Research and Practice 163, 362-372.

Shamsuddin, A.M., Phelps, P.C., Trump, B.F., 1982. Human large intestinal epithelium: light microscopy, histochemistry, and ultrastructure. Human Pathology 13, 790-803.

Sisson, S., Grossman, J.D., Getty, R., 1975. Sisson and Grossman's - The anatomy of the domestic animals, 5th Edition. Saunders, Philadelphia.

Skjolaas, K.A., Burkey, T.E., Dritz, S.S., Minton, J.E., 2006. Effects of *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC) on chemokine and cytokine expression in swine ileum and jejunal epithelial cells. Veterinary Immunollogy and Immunopathology 111, 199-209.

Song, Y., Frey, B., Hampson, D.J., 2012. The use of ELISAs for monitoring exposure of pig herds to *Brachyspira hyodysenteriae*. BMC Veterinary Research 8.

Song, Y., Hampson, D.J., 2009. Development of a multiplex qPCR for detection and quantitation of pathogenic intestinal spirochaetes in the faeces of pigs and chickens. Veterinary Microbiology 137, 129-136.

Specian, R.D., Neutra, M.R., 1982. Regulation of intestinal goblet cell secretion. I. Role of parasympathetic stimulation. American Journal of Physiology 242, G370-379.

Stanton, T. 2006. The Genus *Brachyspira*, In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.) The Prokaryotes. Springer New York, 330-356.

Stanton, T.B., FournieAmazouz, E., Postic, D., Trott, D.J., Grimont, P.A.D., Baranton, G., Hampson, D.J., SaintGirons, I., 1997. Recognition of two new species of intestinal

spirochetes: *Serpulina intermedia* sp. nov. and *Serpulina murdochii* sp. nov. International Journal of Systematic Bacteriology 47, 1007-1012.

Stanton, T.B., Lebo, D.F., 1988. *Treponema hyodysenteriae* growth under various culture conditions. Veterinary Microbiology 18, 177-190.

Stanton, T.B., Rosey, E.L., Kennedy, M.J., Jensen, N.S., Bosworth, B.T., 1999. Isolation, oxygen sensitivity, and virulence of NADH oxidase mutants of the anaerobic spirochete *Brachyspira* (*Serpulina*) *hyodysenteriae*, etiologic agent of Swine Dysentery. Applied Environmental Microbiology 65, 5028-5034.

Stege, H., Jensen, T.K., Moller, K., Baekbo, P., Jorsal, S.E., 2001. Risk factors for intestinal pathogens in Danish finishing pig herds. Preventive Veterinary Medicine 50, 153-164.

Stephens, C.P., Hampson, D.J., 2001. Intestinal spirochete infections of chickens: a review of disease associations, epidemiology and control. Animal Health Research Reviews 2, 83-91.

Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J., 2006. Diseases of swine., 9th Edition. Blackwell Publishing Professional, Ames, Iowa.

Suenaert, P., Bulteel, V., Lemmens, L., Noman, M., Geypens, B., Van Assche, G., Geboes, K., Ceuppens, J.L., Rutgeerts, P., 2002. Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. American Journal of Gastroenterology 97, 2000-2004.

Symonds, D.A., 1974. Paneth cell metaplasia in diseases of the colon and rectum. Archives of Pathology 97, 343-347.

Takeichi, M., 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. Science 251, 1451-1455.

Taylor, D.J., Alexander, T.J., 1971. The production of dysentery in swine by feeding cultures containing a spirochaete. The British Veterinary Journal 127, 58-61.

Taylor, D.J., Blakemore, W.F., 1971. Spirochaetal invasion of the colonic epithelium in swine dysentery. Research in Veterinary Science 12, 177-179.

Taylor, D.J., Simmons, J.R., Laird, H.M., 1980. Production of diarrhoea and dysentery in pigs by feeding pure cultures of a spirochaete differing from *Treponema hyodysenteriae*. Veterinary Records 106, 326-332.

Teige, J., Jr., Nordstoga, K., 1979. Electron microscopic changes in colon in experimental swine dysentery. Acta Veterinaria Scandinavica 20, 224-237.

ter Huurne, A.A., Gaastra, W., 1995. Swine dysentery: more unknown than known. Veterinary Microbiology 46, 347-360.

ter Huurne, A.A., Muir, S., van Houten, M., van der Zeijst, B.A., Gaastra, W., Kusters, J.G., 1994. Characterization of three putative *Serpulina hyodysenteriae* hemolysins. Microbial Pathology 16, 269-282.

Thiagarajah, J.R., Verkman, A.S. 2006. Chapter 72 - Water Transport in the Gastrointestinal Tract, In: Wood, L.R., Johnson, Kim E., Barret, Fayez K., Gishan, Juanita L., Merchant, Hamid M., Said, Jackie D. (Ed.) Physiology of the Gastrointestinal Tract. Academic Press, Burlington, 1827-1845.

Thomson, D., 1914. Controlled Growth en masse (Somatic Growth) of Embryonic Chick Tissue *in vitro*. Procceedings of the Royal Sociecty of Medicine 7, 71-75.

Thomson, J.R., Smith, W.J., Murray, B.P., Murray, D., Dick, J.E., Sumption, K.J., 2001. Porcine enteric spirochete infections in the UK: surveillance data and preliminary investigation of atypical isolates. Animal Health Research Reviews 2, 31-36.

Trott, D.J., Huxtable, C.R., Hampson, D.J., 1996. Experimental infection of newly weaned pigs with human and porcine strains of *Serpulina pilosicoli*. Infection and Immunity 64, 4648-4654.

Trowell, O.A., 1959. The culture of mature organs in a synthetic medium. Experimental Cell Research 16, 118-147.

Turner, J.R., 2009. Intestinal mucosal barrier function in health and disease. Nature Reviews in Immunollogy 9, 799-809.

Vaishnava, S., Behrendt, C.L., Ismail, A.S., Eckmann, L., Hooper, L.V., 2008. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proceedings of the National Academy of Sciences 105, 20858-20863.

Volf, J., Boyen, F., Faldyna, M., Pavlova, B., Navratilova, J., Rychlik, I., 2007. Cytokine response of porcine cell lines to *Salmonella enterica* serovar typhimurium and its *hil*A and *ssr*A mutants. Zoonoses Public Health 54, 286-293.

Waldmann, K.H., Wendt, M., Amtsberg, G., 2000. Investigations on *Brachyspira* - diagnostic and therapeutic strategies in Swine Dysentery. Dtsch Tierarztl Wochenschr 107, 486-489.

Wall, D.M., Nadeau, W.J., Pazos, M.A., Shi, H.N., Galyov, E.E., McCormick, B.A., 2007. Identification of the *Salmonella enterica* serotype typhimurium SipA domain responsible for inducing neutrophil recruitment across the intestinal epithelium. Cell Microbiolology 9, 2299-2313.

Wang, K.S., Ma, T., Filiz, F., Verkman, A.S., Bastidas, J.A., 2000. Colon water transport in transgenic mice lacking aquaporin-4 water channels. American Journal of Physiology 279, G463-470.

Wannemuehler, M.J., Hubbard, R.D., Greer, J.M., 1988. Characterization of the major outer membrane antigens of *Treponema hyodysenteriae*. Infection and Immunity 56, 3032-3039.

Ward, G.E., Winkelman, N.L., 1990. Recognizing the 3 forms of proliferative enteritis in swine. Veterinary Medicine 85, 197-&.

Weinman, E.J., Minkoff, C., Shenolikar, S., 2000. Signal complex regulation of renal transport proteins: NHERF and regulation of NHE3 by PKA. Renal Physiology - American Journal of Physiology 279, F393-399.

Welsh, M.J., Smith, P.L., Fromm, M., Frizzell, R.A., 1982. Crypts are the site of intestinal fluid and electrolyte secretion. Science 218, 1219-1221.

Whipp, S.C., Harris, D.L., Kinyon, J.M., Songer, J.G., Glock, R.D., 1978. Enteropathogenicity testing of *Treponema hyodysenteriae* in ligated colonic loops of swine. American Journal of Veterinary Research 39, 1293-1296.

Whipp, S.C., Pohlenz, J., Robinson, I.M., Harris, D.L. 1982. Pathogenicity of *Treponema hyodysenteriae* in uncontaminated gnotobiotic pigs. In 63rd Annual Meeting of the Conference of Research Workers in Animal Disease (UK), 45-45.

Whipp, S.C., Robinson, I.M., Harris, D.L., Glock, R.D., Matthews, P.J., Alexander, T.J., 1979. Pathogenic synergism between *Treponema hyodysenteriae* and other selected anaerobes in gnotobiotic pigs. Infection and Immunity 26, 1042-1047.

White, R.L., 2001. What *in vitro* models of infection can and cannot do. Pharmacotherapy 21, 292S-301S.

Whiting, R.A., Doyle, L.P., Spray, R.S., 1921a. Swine Dysentery. Bulletin, 3-15.

Whiting, R.A., Spray, R.S., Doyle, L.P., 1921b. Swine Dysentery. Purdue University, 12 p.

Wilberts, B.L., Arruda, P.H., Kinyon, J.M., Madson, D.M., Frana, T.S., Burrough, E.R., 2014. Comparison of sesion severity, distribution, and colonic mucin expression in pigs with acute swine dysentery following oral inoculation with "*Brachyspira hampsonii*" or *Brachyspira hyodysenteriae*. Veterinary Pathology 51, 1096-1108.

Wilcock, B.P., Olander, H.J., 1979. Studies on the pathogenesis of swine dysentery. I. Characterization of the lesions in colons and colonic segments inoculated with pure

cultures or colonic content containing *Treponema hyodysenteriae*. Veterinary Pathology 16, 450-465.

Wlodarska, M., Willing, B., Keeney, K.M., Menendez, A., Bergstrom, K.S., Gill, N., Russell, S.L., Vallance, B.A., Finlay, B.B., 2011. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. Infection and Immunity 79, 1536-1545.

Wood, E.N., Lysons, R.J., 1988. Financial benefit from the eradication of swine dysentery. Veterinary Records 122, 277-279.

Woods, T.A. 1990. Diarrhea, In: Walker, H.K., Hall, W.D., Hurst, J.W. (Eds.) Clinical Methods: The History, Physical, and Laboratory Examinations. Butterworths, Boston, 1087.

Worrell, R.T., Butt, A.G., Cliff, W.H., Frizzell, R.A., 1989. A volume-sensitive chloride conductance in human colonic cell line T84. The American Journal of Physiology 256, C1111-1119.

Xu, J., Gordon, J.I., 2003. Honor thy symbionts. Proceedings of the National Academy of Sciences 100, 10452-10459.

Yu, Q.C., Mergner, W.J., Vigorito, R.D., Resau, J.H., 1990. *Post mortem* viability and early changes in organ-culture of human and rabbit aortic endothelial-cells. Pathobiology 58, 138-145.

Yun, C.H., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C.M., Weinman, E.J., Donowitz, M., 1997. cAMP-mediated inhibition of the epithelial brush border Na+/H+ exchanger, NHE3, requires an associated regulatory protein. Proceedings of the National Academy of Sciences 94, 3010-3015.

Zachrisson, K., Neopikhanov, V., Wretlind, B., Uribe, A., 2001. Mitogenic action of tumour necrosis factor-alpha and interleukin-8 on explants of human duodenal mucosa. Cytokine 15, 148-155.